ELSEVIER

European Journal of Pharmacology 406 (2000) 171–180

ejp

www.elsevier.nl/locate/ejphar

A key role for the mitochondrial benzodiazepine receptor in cellular photosensitisation with δ-aminolaevulinic acid

Maike Mesenhöller, E. Keith Matthews *

Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QJ, UK

Received 25 May 2000; received in revised form 4 August 2000; accepted 18 August 2000

Abstract

The aim of this study was to determine the part played by the mitochondrial benzodiazepine receptor in cellular photosensitisation with the protoporphyrin IX precursor, δ-aminolaevulinic acid. Evaluation of the δ-aminolaevulinic acid-concentration dependence and kinetics of fluorescent protoporphyrin IX accumulation in monolayers of rat AR4 2J pancreatoma cells established a basis for assessing pharmacological modulation of the biosynthetic pathways for protoporphyrin IX production and photocytotoxicity. Iron chelation enhanced the accumulation of photo-active protoporphyrin IX whereas 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide (PK11195), dipyridamole, or 7-(dimethylcarbamoloxy)-6-phenylpyrrolo-[2,1-*d*]benzothiazepine (DPB), competitive ligands of the mitochondrial benzodiazepine receptor, all diminished protoporphyrin IX accumulation, as did acifluorfen, a mitochondrial protoporphyrinogen oxidase inhibitor. In addition to protoporphyrin IX (Em_{max}: 630 nm), δ-aminolaevulinic acid-treated cells also generated a fluorophore of Em_{max} 580 nm; this compound was identified as Zn-protoporphyrin IX. Mitochondrial benzodiazepine receptor ligands increased the formation of the zinc porphyrin whilst decreasing that of protoporphyrin IX. The involvement of the mitochondrial benzodiazepine receptor in the translocation of porphyrins and the formation of Zn-protoporphyrin IX have wide implications for the use of δ-aminolaevulinic acid in photodynamic therapy.

Keywords: Benzodiazepine receptor, mitochondrial; δ-Aminolaevulinic acid; Protoporphyrin IX; Zinc protoporphyrin IX; Aciflourfen; Protoporphyrinogen oxidase

1. Introduction

Porphyrins are naturally occurring compounds present in virtually all cells, some of which, protoporphyrin IX in particular, have potent photodynamic properties. In neoplastic tissue, the accumulation of photoactive protoporphyrin IX within tumour cells can be enhanced via the haem biosynthetic pathway by exogenous δ-aminolaevulinic acid administration (Pottier et al., 1986). This forms the basis of photodynamic therapy, with δ-aminolaevulinic acid-induced protoporphyrin IX producing a photocytotoxic effect via the generation of highly reactive singlet oxygen (Kessel, 1977; Weishaupt et al., 1976). Although the peripheral or mitochondrial benzodiazepine receptor found on the outer mitochondrial membrane remains something of a functional enigma (Gavish et al., 1999), it is a high affinity recognition site for protoporphyrin IX (K_i; 20 nM: Verma and Snyder, 1988), and has been implicated recently in the control of protoporphyrin IX formation, particularly in cells of pancreatic origin (Ratcliffe and Matthews, 1995; Mesenhöller and Matthews, 1998), as well as providing a possible target for photodynamic therapy (Cui and Matthews, 1998; Verma et al., 1998) Furthermore, in many different types of neoplastic cell, the mitochondrial benzodiazepine receptor is now known to display high levels of expression and, hence, binding capacity, but with no change in mitochondrial binding affinity from that of nontransformed cells (Katz et al., 1988, 1990a,b; Venturini et al., 1998).

The aim of this study was therefore to determine the part played by the mitochondrial benzodiazepine receptor in the biosynthetic pathways of protoporphyrin IX production and photosensitisation in rat pancreatoma cells. By evaluation of the δ-aminolaevulinic acid-concentration-dependence and kinetics of fluorescent protoporphyrin IX

* Corresponding author. Tel.: +44-1223-334-032; fax: +44-1223-334-040.

E-mail address: ekm1000@cus.cam.ac.uk (E.K. Matthews).

0014-2999/00/$ - see front matter
PII: S0014-2999(00)00646-4

accumulation in cell monolayers and use of the competitive mitochondrial benzodiazepine receptor ligands, 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide (PK11195) (K_d; 3 nM: Verma and Snyder, 1988), dipyridamole (K_i; 0.68 μM: Hirsch et al., 1988), and 7-(dimethylcarbamoloxy)-6-phenylpyrrolo-[2,1-*d*]benzothiazepine (DPB) (K_i; 9 nM: Fiorini et al., 1994), we show here that the mitochondrial benzodiazepine receptor plays a key role in modulating the synthesis and translocation of porphyrins.

Finally, we have found that when treated with δ-aminolaevulinic acid, pancreatoma cells, in addition to synthesising protoporphyrin IX, also generate a fluorophore with an emission maximum of 580 nm. Chromatographic analysis, fluorimetric characterisation and cation supplementation experiments were carried out to identify this fluorophore; the results of these experiments also are described in this report.

2. Materials and methods

2.1. Chemicals

Foetal bovine serum, 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide (MTT), δ-aminolaevulinic acid, dipyridamole, protoporphyrin IX, poly-D-lysine (lypophilized, sterile and γ-irradiated) and PK11195 were purchased from Sigma (Poole, UK). The protoporphyrin IX fluorescence standards were purchased from Porphyrin Products (P.O. Box 31, Logan, USA). The DPB and zinc protoporphyrin were purchased from Tocris Cookson (Langford, Bristol, UK) and, the acifluorfen was kindly donated by Dr. C. Pearson (Rhône-Poulenc Rorer, UK). All other chemicals and salts were purchased either from Sigma, Fluka (Sigma–Aldrich Chemical, Poole, UK) or BDH (Merck, Lutterworth, UK), with salts having a purity of ≥ 98%.

2.2. Cell culture

AR4 2J cells, derived originally from an azaserine-induced pancreatoma (Longnecker et al., 1979), were maintained as subconfluent monolayers in RPMI 1640 medium containing phenol red, supplemented with 10% of foetal bovine serum, 1% L-glutamine and 1% penicillin–streptomycin. For experimental use, the cells were harvested and resuspended in phenol red and serum-free RPMI 1640 medium.

2.3. Cell survival analysis

For photocytotoxicity experiments, cells were exposed to a range of δ-aminolaevulinic acid concentrations from 1 to 100 μM for 24 h and illuminated for 2–15 min on a modified Sigma T2203 illuminator emitting white light at an intensity of 0.46 mW/cm^2 (12,000 lx). Cell survival was measured spectroscopically 24 h later using the tetrazolium salt, MTT, a method originally developed by Mossmann (1983) and adapted for use on AR4 2J cells by Ratcliffe and Matthews (1995).

2.4. Fluorimetric protoporphyrin IX detection

The protoporphyrin IX content of cell monolayers and extracellular solutions was determined fluorimetrically using a Hitachi F-2000 fluorescence spectrophotometer. Coverslips bearing cell monolayers were held in disposable UV grade cuvettes at 45° to the exciting light beam and emission (λE_m) was measured at 90° with respect to the excitation beam (λE_x, 405 nm). Cell monolayers were produced by seeding 2×10^4 cells ml^{-1} onto sterile, poly-D-lysine coated coverslips (9 × 22 mm; Chance Propper) placed into 35 mm petri dishes (Corning). After 24 h, δ-aminolaevulinic acid was added and, the protoporphyrin IX content of cell monolayers and of the medium measured either at different times after the addition of a fixed amount of δ-aminolaevulinic acid (100 μM), or 24 h after the addition of a range of δ-aminolaevulinic acid concentrations (0–500 μM). The magnitude of protoporphyrin IX fluorescence was measured at 630 nm and an additional fluorophore at 580 nm; these values were not corrected for the small levels of cellular or medium autofluorescence seen in the absence of δ-aminolaevulinic acid.

2.5. Fluorescence measurement by fluorescence plate reader

A fluorescence plate reader (Fluoro Count™, Canberra Packard) with excitation (λE_x) of 400 nm (band width, 35 nm) and emission (λE_m) at 620 nm (band width, 35 nm) was also used for porphyrin measurement. In these experiments, 5×10^5 cells were distributed into each well of a 96-well ViewPlate (black, clear bottom, tissue culture treated; Canberra Packard). δ-Aminolaevulinic acid (250 μM) was added after 24 h and, the fluorescence was measured at timed intervals from 2 to 24 h. To study the effects of desferroxamine, 20 or 50 μM, the mitochondrial benzodiazepine receptor ligands isoquinoline carboxamide (PK11195), 10 or 20 μM, and the pyrrolo compound DPB, 20 μM, or the protoporphyrinogen inhibitor acifluorfen, 20 μM, the cells were preincubated with the modulator for 24 h before addition of the δ-aminolaevulinic acid.

2.6. High performance liquid chromatography (HPLC)

HPLC was used for porphyrin analysis (Luo and Lim, 1995). The wavelength detector of the HPLC system was set at the absorption maximum for authentic protoporphyrin IX and Zn-protoporphyrin IX (λmax, 404 nm). Gradient mixtures of ammonium acetate (1 M, pH 5.5)/acetonitrile (9:1) (solvent A) and methanol/acetonitrile

(9:1) (solvent B) were used. The column was re-equilibrated with 100% solvent A for 10 min before sample injection and protoporphyrin IX and Zn-protoporphyrin IX standards were run before and after each sample.

2.7. Acetone-ammonium hydroxide extraction of the cells

Cells for HPLC analysis were treated with either 100 μM δ-aminolaevulinic acid alone, δ-aminolaevulinic acid together with PK11195, 20 μM, or with zinc acetate, 10 μM. After 24 h, the cells were homogenised in 2 ml of acetone containing 0.1 M ammonium hydroxide. Precipitated protein was removed by centrifugation and resuspended in phosphate-buffered saline (1 ml) for protein determination using the BioRad protein assay (Bradford, 1976). The fluorescence spectra of the acetone-ammonium hydroxide extracts were also determined using quartz cuvettes (4.5 ml, Hellma), scanning the emission wavelength from 500 to 700 nm ($E_x = 405$ nm).

2.8. Statistical analysis

All values quoted are means ± S.E.M. Statistical significance was determined by the two-tailed, unpaired Student's *t*-test and values of $P \leq 0.05$ were considered to be statistically significant.

3. Results

3.1. δ-Aminolaevulinic acid-induced photocytotoxicity

δ-Aminolaevulinic acid-mediated phototoxicity produced a sigmoidal dose-dependent reduction in cell survival, as measured by the MTT assay, where the efficacy was dependent on the light dose (i.e., light intensity and duration). At an intensity of 0.47 mW/cm^2, illumination times of 2 or 4 min gave submaximal photocytotoxicity for δ-aminolaevulinic acid (EC_{50} values of 28.6 ± 5.3 μM and 21.3 ± 4.9 μM, respectively; $n = 6$). Maximal cytotoxicity was achieved at all illumination times ≥ 8 min, the EC_{50} being decreased to 19.2 ± 1.2 μM δ-aminolaevulinic acid ($n = 6$).

3.2. Concentration-dependence and kinetics of δ-aminolaevulinic acid-induced protoporphyrin IX fluorescence

The strong fluorescence signature of the effector molecule protoporphyrin IX allowed the concentration and time dependence of its intracellular accumulation to be determined since there is a linear relationship between the concentration and fluorescence (E_m, 630 nm) of standard solutions of protoporphyrin IX (data not shown).

Cellular fluorescence was evaluated by spectral scanning fluorimetry after treatment with δ-aminolaevulinic acid (Fig. 1). Measurements of cell monolayer fluorescence at 630 nm showed a steep dose–response relationship for δ-aminolaevulinic acid, reaching a plateau at δ-aminolaevulinic acid concentrations > 250 μM, the fluorescence value increasing from a basal level of 0.16 ± 0.03 to 3.98 ± 0.3 ($n = 6$). Some extracellular accumulation of protoporphyrin IX was also seen. In contrast, whereas there was a small increase in the fluorophore peaking at 580 nm (from 0.15 ± 0.04 to 0.45 ± 0.02) in cell monolayers, there was a larger increase in the 580 nm fluorescence of medium, from 0.4 ± 0.04 in the absence, to 2.86 ± 0.24 in the presence of, 500 μM δ-aminolaevulinic acid ($n = 6$). Cellular protoporphyrin IX fluorescence increased linearly over the 24 h period reaching a final fluorescence value of 2.01 ± 0.26 together with an in-

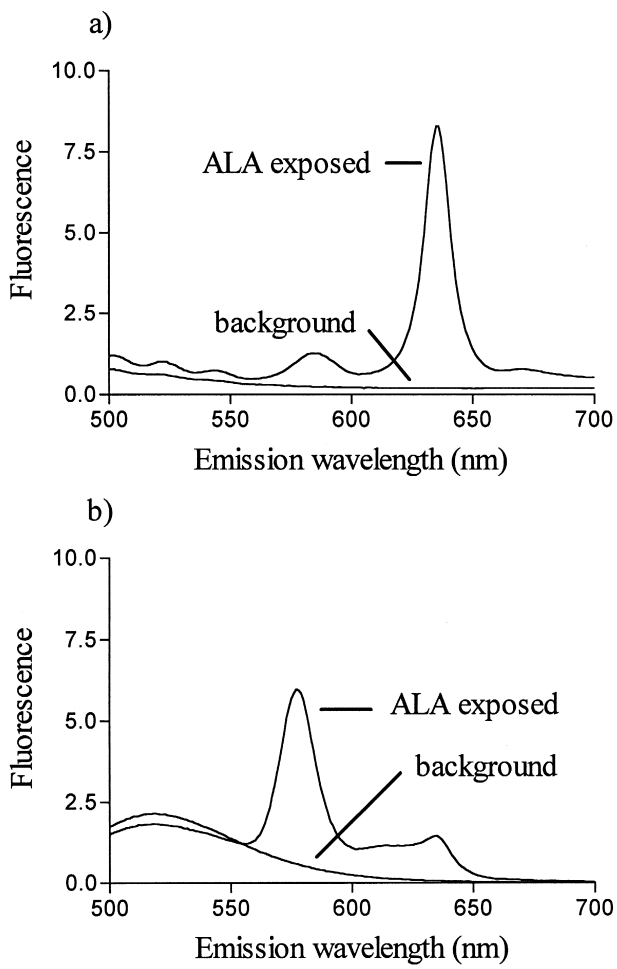


Fig. 1. Characteristic wavelength scans for δ-aminolaevulinic acid-induced protoporphyrin IX fluorescence: Wavelength scans obtained from (a) cell monolayers and (b) culture medium showing the background autofluorescence of the cells and medium and the fluorescence developed after a 24-h incubation with 250 μM δ-aminolaevulinic acid. The fluorescence at 630 nm is attributable to protoporphyrin IX whereas the 580-nm fluorescence may be due to a protoporphyrin IX derivative (see text). Excitation at 405 nm.

crease in the signal at 580 nm to 0.29 ± 0.04 ($n = 6$). The medium fluorescence of both protoporphyrin IX and the compound fluorescing at 580 nm also increased with time, reaching, after 24 h, values of 0.47 ± 0.09 and 1.64 ± 0.19 for the 630 and 580 nm signal, respectively ($n = 6$). These results indicate that the mechanisms responsible for the conversion of δ-aminolaevulinic acid to protoporphyrin IX are operating at a submaximal capacity when the cells are exposed to < 250 μM δ-aminolaevulinic acid.

3.3. Involvement of the mitochondrial benzodiazepine receptor in concentration-dependent δ-aminolaevulinic acid-induced protoporphyrin IX accumulation

To determine the role of the mitochondrial benzodiazepine receptor in protoporphyrin IX production, the effect of the competitive mitochondrial benzodiazepine receptor ligand PK11195 (20 μM) on the concentration-dependent accumulation of protoporphyrin IX fluorescence was examined. Table 1 shows a significant reduction ($P < 0.005$) by PK11195 in the intracellular protoporphyrin IX fluorescence at 630 nm, an effect which was seen at all concentrations of δ-aminolaevulinic acid used. A similar action was exerted by an alternative mitochondrial benzodiazepine receptor ligand, dipyridamole (20 μM), which also significantly decreased the cellular protoporphyrin IX fluorescence induced by 100 μM δ-aminolaevulinic acid (from 2.01 ± 0.26 to 0.58 ± 0.14; $P < 0.005$). In addition to the effects of PK11195 on intracellular protoporphyrin IX accumulation, a decrease in the medium fluorescence of protoporphyrin IX at 630 nm in the presence of PK11195 was also observed, with the changes becoming significant at δ-aminolaevulinic acid concentrations ≥ 50 μM (see Table 1).

Interestingly, the compound fluorescing at 580 nm also increased progressively in the cell monolayers with increasing δ-aminolaevulinic acid concentration, particularly in the presence of PK11195. However, the effect of PK11195 on the cellular 580-nm fluorescence only became significant ($P < 0.01$) at the higher concentrations of δ-aminolaevulinic acid used (i.e., 250 μM). In contrast, at δ-aminolaevulinic acid concentrations greater than 50 μM, the medium fluorescence of the precursor or metabolite fluorescing at 580 nm decreased significantly in the presence of PK11195, implying that the mitochondrial benzodiazepine receptor is involved in both the production of protoporphyrin IX and the fluorescence signal at 580 nm. Experiments to identify the compound responsible for the 580-nm fluorescence are described below (Section 3.5).

3.4. Modulation of the haem biosynthetic pathway

For the modulation of the haem biosynthetic pathway, the comparative effects of iron chelation, mitochondrial benzodiazepine receptor ligands, and protoporphyrinogen oxidase inhibition were analysed using the fluorescence well plate technique (see Methods). The iron chelator desferroxamine significantly increased the protoporphyrin IX levels present in AR4 2J cells upon δ-aminolaevulinic acid (250 μM) treatment for 4 h or more ($P < 0.01$) (see Fig. 2(a)).

The effects of competitive mitochondrial benzodiazepine receptor binding by DPB (20 μM) and PK11195 (20 μM) are shown in Fig. 2(b). Preincubation with DPB or PK11195 decreased ($P < 0.01$) the δ-aminolaevulinic acid-induced protoporphyrin IX fluorescence after 6 h, with a greater effect after 24 h ($P < 0.005$). Interestingly, analysis of the medium fluorescence showed a marked increase of the 580-nm fluorophore in the presence of PK11195 (see Table 1), again implicating the mitochondrial benzodiazepine receptor in the production of this signal.

Table 1
Effect of PK11195 on the δ-aminolaevulinic acid concentration-dependence of PPix accumulation over a 24-h incubation period: The comparative effects of the mitochondrial benzodiazepine receptor ligand PK11195 (PK) on the δ-aminolaevulinic acid-induced fluorescence in cell monolayers and the supernatant medium are given

Concentration of δ-aminolaevulinic acid (μM)	Sample evaluated	Fluorescence at 630 nm		Fluorescence at 580 nm	
		control	+20 μM PK	control	+20 μM PK
None	cells	0.16 ± 0.02	0.17 ± 0.02	0.17 ± 0.02	0.19 ± 0.02
	medium	0.11 ± 0.03	0.10 ± 0.01	0.43 ± 0.03	0.44 ± 0.08
50	cells	1.46 ± 0.26	0.19 ± 0.01[b]	0.33 ± 0.02	0.35 ± 0.01
	medium	0.25 ± 0.07	0.11 ± 0.01[a]	0.66 ± 0.06	0.55 ± 0.09
100	cells	2.41 ± 0.40	0.32 ± 0.06[b]	0.42 ± 0.03	0.41 ± 0.02
	medium	0.33 ± 0.08	0.07 ± 0.02[a]	1.01 ± 0.09	0.47 ± 0.03[b]
250	cells	3.22 ± 0.54	0.62 ± 0.11[b]	0.45 ± 0.02	0.81 ± 0.18[a]
	medium	0.37 ± 0.05	0.08 ± 0.01[b]	2.10 ± 0.31	0.57 ± 0.05[b]
500	cells	3.67 ± 0.33	1.47 ± 0.26[b]	0.50 ± 0.02	0.94 ± 0.17[b]
	medium	0.42 ± 0.06	0.13 ± 0.02[b]	2.08 ± 0.47	0.76 ± 0.11[b]

The data was obtained fluorimetrically, but the background autofluorescence of the cells was not subtracted from the overall fluorescence. Values given are means ± S.E.M. from ≥ 6 experiments.

[a] Denotes $P < 0.01$, when compared with control.

[b] Denotes $P < 0.005$, when compared with control.

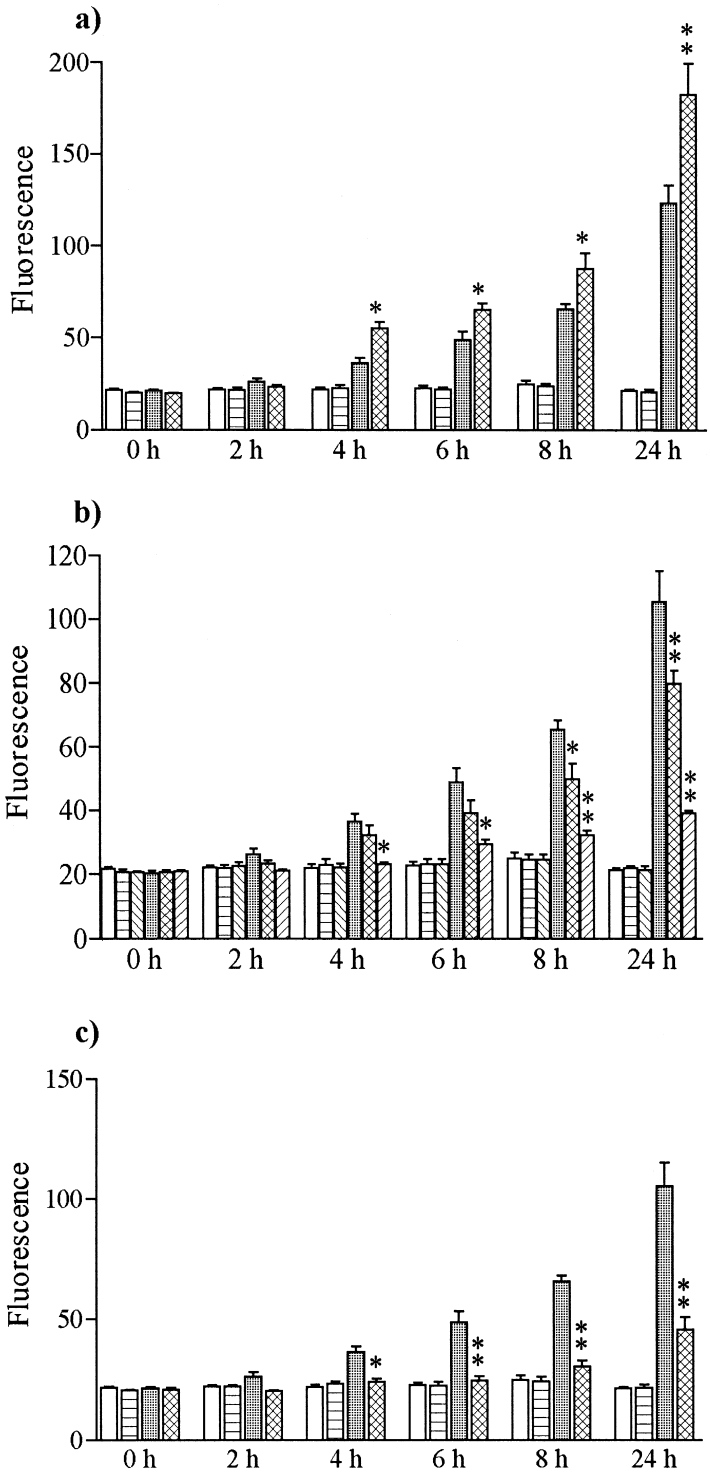
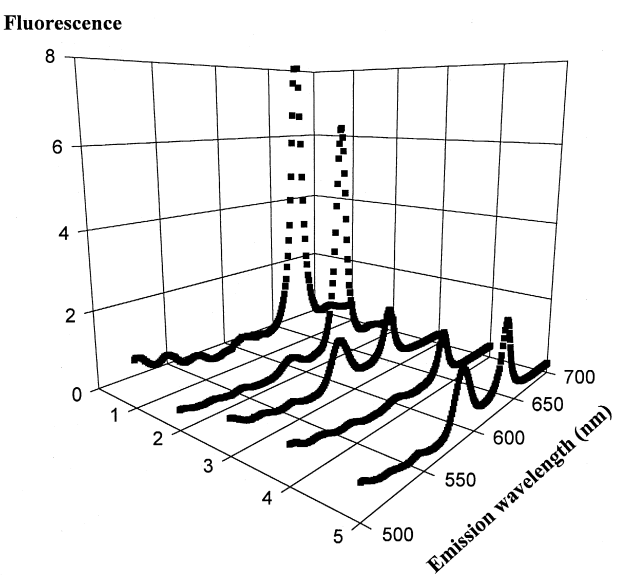


Fig. 2. Effect of iron chelation, mitochondrial benzodiazepine receptor ligands and protoporphyrinogen oxidase inhibition on δ-aminolaevulinic acid-induced protoporphyrin IX fluorescence. Fluorescence was measured with a fluorescence plate-reader at timed intervals in cells without δ-aminolaevulinic acid (□) and after the addition of δ-aminolaevulinic acid, 250 μM (dot-filled square). In (a), this fluorescence was compared with that of cells incubated with desferroxamine, 20 μM, in control cells (▤) and in cells after addition of δ-aminolaevulinic acid, 250 μM (cross-filled square). In (b), cells were incubated with DPB, 20 μM, in the absence (▤), or presence of δ-aminolaevulinic acid, 250 μM (cross-filled square); or with PK11195, 20 μM, in the absence (▽) or presence of δ-aminolaevulinic acid, 250 μM (△). In (c), cells were incubated with the protoporphyrinogen oxidase inhibitor acifluorfen, 50 μM, in the absence (▤) or the presence of δ-aminolaevulinic acid, 250 μM (cross-filled square). Significant changes due to the modulators in the presence of δ-aminolaevulinic acid are denoted by * for $P \leq 0.01$ and ** for $P \leq 0.005$. Data values are means ± S.E.M. ($n = 5$).

Finally, acifluorfen was used to characterise the action of a protoporphyrinogen oxidase inhibitor on δ-aminolaevulinic acid-induced protoporphyrin IX accumulation. Incubation with 20 μM acifluorfen prior to exposure to 250 μM δ-aminolaevulinic acid caused a significant decrease ($P < 0.01$) in protoporphyrin IX accumulation at incubation times ≥ 4 h (Fig. 2(c)).

3.5. Identification of the 580-nm fluorescence signal

To test whether the 580-nm fluorescent signal observed in the medium of δ-aminolaevulinic acid-treated cells is attributable to the extracellular formation of a protoporphyrin IX derivative, the cells were exposed to exogenous protoporphyrin IX (10 μM). Whereas in three such experiments, the cellular fluorescence of protoporphyrin IX was up to 10-fold greater than that of the medium after 24 h, there was, in contrast, little or no fluorescence signal at 580 nm in the medium or the cells over the 24-h period (data not shown). Alternatively, the signal may arise from the intracellular formation of a metal chelate of protoporphyrin IX. In further experiments therefore cells were exposed to various metal ions (Mg^{2+}, Zn^{2+} and Fe^{3+}) or PK11195 to study their effects on δ-aminolaevulinic acid-induced protoporphyrin IX fluorescence (see Fig. 3). The inset table indicates the ratio between the fluorescence signals at 580 and 630 nm. Incubation with δ-aminolaevulinic acid alone resulted in the appearance of a fluorescence peak at 630 nm, attributable to protoporphyrin IX. Whereas magnesium has little or no effect on the fluorescence spectrum, a significant shift was seen in the presence of zinc, which decreased fluorescence at 630 nm and increased at 580 nm. The presence of ferrous iron reduced the protoporphyrin IX signal (630 nm) almost to control levels, indicating the conversion of protoporphyrin IX to nonfluorescent haem. Finally, as indicated previously (see Fig. 2), 20 μM PK11195 decreased the fluorescence signal attributable to protoporphyrin IX, and increased the fluorescence peak at 580 nm. This peak correlates with the peak of the fluorescence signal seen in cells preincubated with zinc, indicating that the 580-nm fluorophore might be due to a Zn chelate of protoporphyrin IX, formed within the cells.



Sample	Peak ratio 580 nm : 630 nm
1) ALA only	1 : 10.5
2) Mg^{2+} + ALA	1 : 9.6
3) Zn^{2+} + ALA	1 : 1.1
4) Fe^{2+} + ALA	1 : 2.6
5) PK11195 + ALA	1 : 1.3

Fig. 3. Fluorescence of protoporphyrin IX in cell monolayers following the addition of metal ions or mitochondrial benzodiazepine receptor ligand. Shown are the fluorescence spectra of cells incubated with (1) δ-aminolaevulinic acid (ALA), 100 μM alone for 24 h and the effect of 24-h pre-incubation with either, (2) 100 μM magnesium, Mg^{2+}; (3) zinc, Zn^{2+}; (4) ferrous iron, Fe^{2+}, or (5) PK11195, 20 μM, before the addition of δ-aminolaevulinic acid (ALA), 100 μM. The ratios between the peaks at 580 nm and 630 nm are given in the inset table.

HPLC analysis was carried out to further identify the 580-nm fluorophore by characterising cellular porphyrin content. The HPLC elution profile of standard Zn-protoporphyrin IX and protoporphyrin IX showed elution maxima at 18.69 and 19.79 min, respectively (data not shown). HPLC analysis of alkaline acetone-extracted control cells, cells treated with 100 μM δ-aminolaevulinic acid alone, or cells preincubated with 100 μM Zn-acetate or 20 μM PK11195 before δ-aminolaevulinic acid treatment, was carried out and the results are shown in Fig. 4. Panel (a) shows the HPLC record obtained from control cells, containing only basal levels of protoporphyrin IX. Incubation with δ-aminolaevulinic acid (100 μM for 24 h) resulted in an increase of protoporphyrin IX, as indicated by the greater magnitude of the 19.69-min eluate (panel b). A preceding peak eluting at 18.79 min also appears, representative of Zn-protoporphyrin IX. Panel (c) shows the effect of PK11195 (20 μM) treatment. Even though the amplitude of the peaks is decreased, partly due to the effect of PK11195 on protoporphyrin IX accumulation and partly due to a lower protein content of this sample (45 μg ml^{-1} compared to 65 μg ml^{-1} in panel c), the ratio of the Zn-protoporphyrin IX peak increases from 35.7% of the protoporphyrin IX eluate in the δ-aminolaevulinic acid treated sample to 44.4% in the presence of PK11195, which correlates with the results obtained from the fluorescence studies. Finally, cells were exposed to zinc (100 μM) for 24 h before δ-aminolaevulinic acid treatment (100 μM δ-aminolaevulinic acid). The resulting eluate is shown in panel (d). Here, the amplitude of the peaks eluting at 18.79 and 19.69 min is reversed, the Zn-protoporphyrin IX peak now being 255% of the size of the protoporphyrin IX peak. This confirms that the peak eluting at 18.79 min is due to Zn-protoporphyrin IX, as indicated by the standard porphyrins.

Finally, the HPLC elution peaks at 18.79 and 19.69 min were correlated to fluorescence measurements by analysis of the fluorescence of the same cellular acetone extracts as

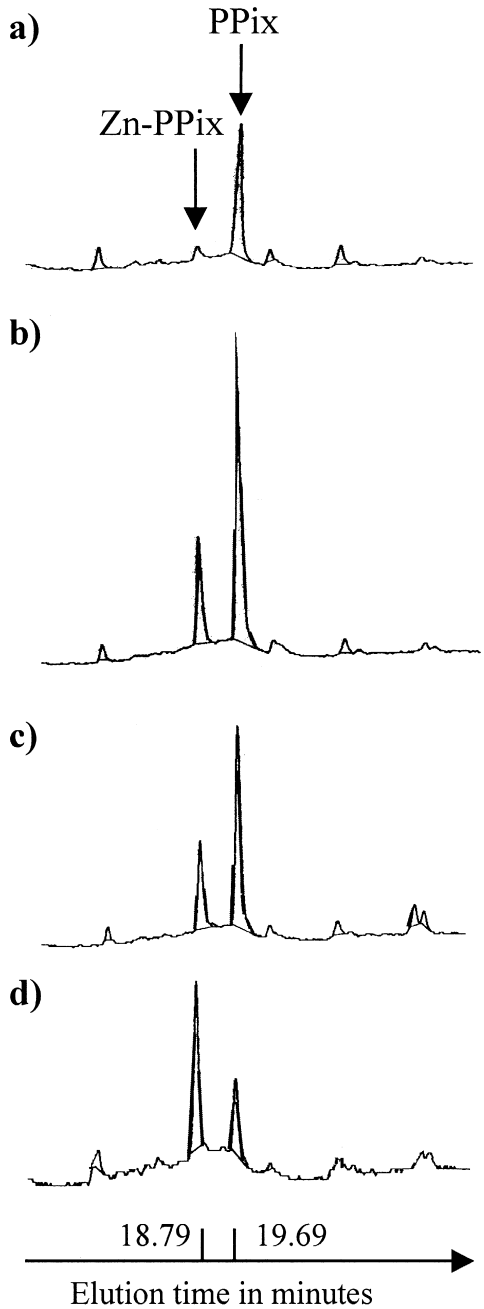


Fig. 4. HPLC analysis of cellular porphyrin extracts: HPLC records obtained from untreated control cells (a); cells incubated with 100 μM δ-aminolaevulinic acid (b); effects of PK11195 (c) and zinc (d) pre-treatment before δ-aminolaevulinic acid administration are shown. The peaks eluting at 18.79 min and 19.96 min correspond to Zn-protoporphyrin IX and protoporphyrin IX, respectively.

those used for HPLC analysis. The fluorescence spectra confirmed the presence of two peaks corresponding to those illustrated in Fig. 3, i.e., at 580 and 630 nm.

4. Discussion

These results demonstrate that photosensitisation of rat pancreatoma cells occurs following exposure to δ-aminolaevulinic acid and that the extent of photocytotoxicity is dependent on light dose. δ-Aminolaevulinic acid-mediated photosensitisation relies on the production of photoactive protoporphyrin IX, the immediate precursor of haem. The haem biosynthetic pathway involves mitochondrial membrane transport systems as well as several cytoplasmic and mitochondrial enzymatic transformations. It is known that selective accumulation of protoporphyrin IX in tumour cells is due to a decrease in ferrochelatase activity, and overexpression of PBG deaminase, the second rate limiting step of the haem biosynthetic pathway (Rubino and Rasetti, 1966; Van Hillegersberg et al., 1992). We have demonstrated in the present study that iron chelation can further enhance protoporphyrin IX accumulation by preventing the formation of haem, a finding supported by observations in other cell lines and in vivo (Berg et al., 1996; Chang and Bown, 1997; Fijan et al., 1995).

For haem and its precursors to cross the mitochondrial membrane, it is likely that several recognition sites are involved in porphyrin transport. One such site with a high affinity for protoporphyrin IX is the mitochondrial (peripheral) benzodiazepine receptor, which is associated with a voltage-dependent anion channel (VDAC) (Verma et al., 1987), and may therefore play an important role in controlling the intramitochondrial route of porphyrin products.

We have previously reported (Ratcliffe and Matthews, 1995) that competitive mitochondrial benzodiazepine receptor ligands such as the isoquinoline carboxamide PK11195 exert a photoprotective effect, presumably by displacing any endogenous protoporphyrin IX from the mitochondrial benzodiazepine receptor binding site. However, it was not known whether this effect was due to protection of the mitochondrial benzodiazepine receptor from photodamage, a decrease in the translocation of protoporphyrin IX precursors or metabolites across the mitochondrial membrane, or some other mechanism. Not only do the present results demonstrate that the mitochondrial benzodiazepine receptor plays a key role in the translocation of protoporphyrin IX precursors and protoporphyrin IX itself across the mitochondrial membrane, but that mitochondrial benzodiazepine receptor ligands also diminish the accumulation of photo-active protoporphyrin IX. Summarised in Fig. 5 are the factors responsible for photosensitisation following δ-aminolaevulinic acid treatment, showing clearly the strategic location of the mitochondrial benzodiazepine receptor. Taken together with the recent demonstration that the mitochondrial benzodiazepine receptor is involved in the translocation of coproporphyrinogen III across the mitochondrial membrane (Taketani et al., 1994 and 1995), our results point to a key role for the mitochondrial benzodiazepine receptor in the operation of the haem biosynthetic pathway. Competitive inhibition of the mitochondrial benzodiazepine receptor by PK11195, dipyridamole or DPB, therefore decreases protoporphyrin IX accumulation by preventing the transport of porphyrins across the mitochondrial membrane.

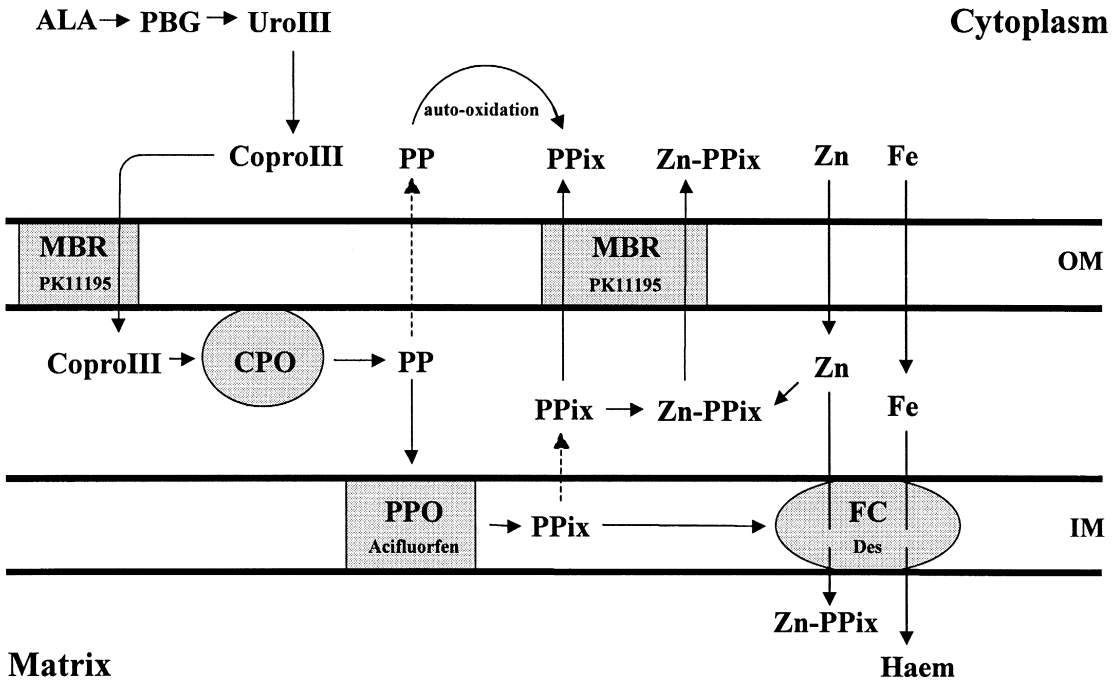


Fig. 5. Mitochondrial benzodiazepine receptor (MBR) and the haem biosynthetic pathway: The sites of action of mitochondrial benzodiazepine receptor ligands (e.g. PK11195), a protoporphyrinogen oxidase inhibitor (Aciflourfen), and iron chelator (Des: desferroxamine) are shown in relation to mitochondrial localisation and involvement in δ-aminolaevulinic acid-induced photosensitisation. Possible routes of zinc (Zn) uptake, the formation of Zn-protoporphyrin IX (Zn-PPix), and its translocation across mitochondrial membranes are illustrated, where OM indicates the outer, and IM the inner, mitochondrial membrane; also indicated are δ-aminolaevulinic acid, ALA; porphobilinogen, PBG; Uroporphyrinogen III, UroIII; Coproporphyrinogen III, CoproIII; coproporphyrinogen oxidase, CPO; protoporphyrinogen, PP; protoporphyrinogen oxidase, PPO; protoporphyrin IX, PPix; iron, Fe; and ferrochelatase, FC.

These results also indicate that the mitochondrial benzodiazepine receptor plays an important role in porphyrin metabolism per se as well as being implicated in δ-aminolaevulinic acid-mediated photosensitisation.

Protoporphyrinogen oxidase is the penultimate enzyme of the haem biosynthetic pathway catalysing the oxidation of protoporphyrinogen IX to protoporphyrin IX within the mitochondria (Fig. 5). We therefore used acifluorfen, a diphenyl ether that closely resembles sterically one half of the protoporphyrin molecule (Nandihalli and Duke, 1994), to target this enzyme and found a significant decrease in protoporphyrin IX fluorescence. The main route for protoporphyrin IX production is therefore likely to be via mitochondrial coproporphyrinogen oxidase and protoporphyrinogen oxidase, with little or no protoporphyrin IX arising via the auto-oxidation of cytoplasmic protoporphyrinogen, see Fig. 5.

Our fluorescence studies have revealed that in pancreatoma cells a compound with a peak fluorescence at 580 nm appeared together with protoporphyrin IX, and was particularly prominent following exposure to high concentrations of δ-aminolaevulinic acid, and in the presence of mitochondrial benzodiazepine receptor ligands, e.g. PK11195. Initially it was thought that this fluorophore might be either a precursor of protoporphyrin IX and haem or a protoporphyrin IX metabolite. Alternatively, the additional fluorophore might result from the insertion of a metal other than Fe^{2+} into the protoporphyrin macrocycle. Administration of various trace metals to study their effects on δ-aminolaevulinic acid-induced fluorescence showed that extracellular supplementation with zinc yielded a cellular fluorescence peak at 580 nm. This peak correlated closely with the peak observed in pancreatoma cells treated with δ-aminolaevulinic acid and the mitochondrial benzodiazepine receptor inhibitor PK11195, and therefore indicates that the 580-nm signal may be due to the formation of a Zn-chelated porphyrin molecule. This hypothesis is supported by the fact that zinc protoporphyrin is synthesised enzymatically by ferrochelatase in some cell types, especially when iron concentrations are limiting (Jacobs et al., 1998). The identity of the unknown 580-nm fluorescence compound was further confirmed by HPLC analysis of cellular extracts, as well as by parallel fluorescence measurements of the same samples. All these results are consistent with the conclusion that the fluorescence at 580 nm seen upon treatment with high δ-aminolaevulinic acid concentrations or upon preincubation with mitochondrial benzodiazepine receptor inhibitors is due to the insertion of zinc into the porphyrin ring structure, especially since the pancreas is known to have a high zinc content. The lower pH seen in many types of cancerous tissue could also favour the insertion of zinc because the optimal pH

for ferrochelatase activity lies at about 8.0, whereas the Zn-chelatase activity of the same enzyme has a pH optimum of 5.5 (Nunn et al., 1988).

Identification of the 580-nm fluorescence signal as Zn-protoporphyrin IX suggests also that the mitochondrial benzodiazepine receptor might be implicated not only in protoporphyrin IX transport and production, but could also influence the ion content of mitochondria, favouring the insertion of zinc into the porphyrin ring structure since it is known that Zn^{2+} has a high affinity for the mitochondrial Ca^{2+} uniporter (Saris and Niva, 1994). The interplay of zinc with the haem biosynthetic pathway is also illustrated in Fig. 5. In future experiments, it will be important not only to measure the total cellular content of Zn-protoporphyrin IX but to determine also whether Zn-protoporphyrin IX is localised to distinct cellular compartments.

To conclude, we have characterised the concentration dependence and kinetics of δ-aminolaevulinic acid-mediated photosensitisation of pancreatoma cells and illustrated the key role played by the mitochondrial benzodiazepine receptor in photosensitisation, as well as pinpointing two sites of enzyme modulation; thus, while iron chelation enhances protoporphyrin IX accumulation in the tumour cells, the protoporphyrinogen oxidase inhibitor acifluorfen decreases protoporphyrin IX synthesis. Finally, identification of the 580-nm fluorescence signal as Zn-protoporphyrin IX has raised the important possibility that although δ-aminolaevulinic acid-induced photocytotoxicity is due mainly to the accumulation of protoporphyrin IX within the cells, this action may be modulated by the insertion of zinc into the porphyrin structure. The involvement of the mitochondrial benzodiazepine receptor and the formation of Zn-protoporphyrin IX have wide implications for the use of δ-aminolaevulinic acid in photodynamic therapy.

Acknowledgements

We thank Mrs. Margaret Forsyth for expert technical assistance and Dr. Ross Boyle, Department of Biological Science, University of Essex, for the use of HPLC equipment.

References

Berg, K., Anholt, H., Bech, O., Moan, J., 1996. The influence of iron chelators on the accumulation of protoporphyrin IX in 5-aminolevulinic acid-treated cells. Br. J. Cancer 74, 688–697.

Bradford, M.M., 1976. A rapid and sensitive method for the determination of microgram quantities of protein utilising the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Chang, S.C., Bown, S.G., 1997. Photodynamic therapy: applications in bladder cancer and other malignancies. J. Formosan Med. Assoc. 96, 853–856.

Cui, Z.-J., Matthews, E., 1998. Photodynamic modulation of cellular function. Acta Pharmacol. Sinica 19, 297–303.

Fijan, S., Honigsmann, H., Ortel, B., 1995. Photodynamic therapy of epithelial skin tumours using delta-aminolaevulinic acid and desferroxamine. Br. J. Dermatol. 133, 282–288.

Fiorini, I., Nacci, V., Ciani, S.M., Garafalo, A., Campiani, G., Savini, L., Novellino, E., Greco, G., Bernasconi, P., Mennini, T., 1994. Novel ligands specific for mitochondrial benzodiazepine receptors: 6-Arylpyrrolo[2,1-*d*][1,5]benzothiazepine derivatives. Synthesis, structure-activity relationships and molecular modelling studies. J. Med. Chem. 37, 1427–1438.

Gavish, M., Bachman, I., Shoukrun, R., Katz, Y., Veenman, L., Weisinger, G., Weizman, A., 1999. Enigma of the peripheral benzodiazepine receptor. Pharmacol. Rev. 51, 629–650.

Hirsch, J.D., Beyer, C.F., Malkowitz, L., Loullis, C.C., Blume, A.J., 1988. Characterization of ligand binding to mitochondrial benzodiazepine receptors. Mol. Pharmacol. 34, 164–172.

Jacobs, J.M., Sinclair, P.R., Sinclair, J.F., Gorman, N., Walton, H.S., Wood, S.G., Nichols, C., 1998. Formation of zinc protoporphyrin in cultured hepatocytes: effects of ferrochelatase inhibition, iron chelation or lead. Toxicology 125, 95–105.

Katz, Y., Eitan, A., Amiri, Z., Gavish, M., 1988. Dramatic increase in peripheral benzodiazepine binding sites in human colonic adenocarcinoma as compared to normal colon. Eur. J. Pharmacol. 148, 483–484.

Katz, Y., Ben-Baruch, G., Kloog, Y., Menczer, J., Gavish, M., 1990a. Increased density of peripheral benzodiazepine-binding sites in ovarian carcinomas as compared with benign ovarian tumours and normal ovaries. Clin. Sci. 78, 155–158.

Katz, Y., Eitan, A., Gavish, M., 1990b. Increase in peripheral benzodiazepine binding sites in colonic adenocarcinoma. Oncology 47, 139–142.

Kessel, D., 1977. Effects of photoactivated porphyrins at the cell surface of leukaemia L1210 cells. Biochemistry 16, 3443–3449.

Longnecker, D.S., Lilja, H.S., French, J., Kuhlmann, E., Noll, W., 1979. Transplantation of azaserine-induced carcinomas of the pancreas in rats. Cancer Lett. 7, 197–202.

Luo, J.L., Lim, C.K., 1995. Isolation and characterisation of new porphyrin metabolites in human porphyria–cutanea–tarda and in rats treated with dexachlorobenzene by HPTLC, HPLC and liquid secondary-ion mass spectrometry. Biomed. Chromatogr. 9, 113–122.

Mesenhöller, M., Matthews, E., 1998. Photosensitisation of pancreatic tumour cells: effects of mitochondrial benzodiazepine receptor ligands. Br. J. Pharmacol. 123, 134 pp.

Mossmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: an application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Nandihalli, U.B., Duke, S.O., 1994. Structure-activity-relationships of protoporphyrinogen oxidase inhibiting herbicides. ACS Symp. Ser. 559, 133–146.

Nunn, A.V., Norris, W.P., Hawk, J.L.M., Cox, T.M., 1988. Zinc chelatase in human lymphocytes: detection of the enzymatic defect in erythropoietic protopophyria. Anal. Biochem. 174, 146–150.

Pottier, R.H., Chow, Y., Laplante, J., Truscott, T.G., Kennedy, J.C., 1986. Non invasive technique for obtaining fluorescence excitation and emission spectra in vivo. Photochem. Photobiol. 44, 679–687.

Ratcliffe, S.L., Matthews, E.K., 1995. Modification of the photodynamic action of δ-aminolaevulinic acid on rat pancreatoma cells by mitochondrial benzodiazepine receptor ligands. Br. J. Cancer 71, 300–305.

Rubino, G.F., Rasetti, L., 1966. Porphyrin metabolism in human neoplastic tissues. Panminerva Med. 8, 290–292.

Saris, N.E., Niva, K., 1994. Is Zn^{2+} transported by the mitochondrial uniporter? FEBS Lett. 336, 195–198.

Taketani, S., Kohno, H., Okuda, M., Furukawa, T., Tokunaga, R., 1994. Induction of peripheral-type benzodiazepine receptors during differentiation of mouse erythroleukemia cells: possible involvement of these receptors in haem biosynthesis. J. Biol. Chem. 269, 7527–7531.

Taketani, S., Kohno, H., Furukawa, T., Tokunaga, R., 1995. Involvement of peripheral-type benzodiazepine receptors in the intracellular transport of haem and porphyrins. J. Biochem. 117, 875–880.

Van Hillegersberg, R., Van den Berg, J.W.O., Kort, W.J., Terpstra, O.T., Wilson, J.H.P., 1992. Selective accumulation of endogenously produced porphyrins in a liver metastasis model in rats. Gastroenterology 103, 647–651.

Venturini, I., Zeneroli, M.L., Corsi, L., Avallone, R., Farina, F., Alho, H., Baraldi, C., Ferrarese, C., Pecora, N., Frigo, M., Ardizzone, G., Arrigo, A., Pellici, R., Baraldi, M., 1998. Up-regulation of peripheral benzodiazepine receptor system in hepatocellular carcinoma. Life Sci. 63, 1269–1280.

Verma, A., Snyder, S.H., 1988. Characterisation of porphyrin interactions with peripheral-type benzodiazepine receptors. Mol. Pharmacol. 34, 800–805.

Verma, A., Ney, J.S., Snyder, S., 1987. Porphyrins are endogenous ligands for the mitochondrial peripheral-type benzodiazepine receptor. Proc. Natl. Acad. Sci. U. S. A. 84, 2256–2260.

Verma, A., Facchina, S.L., Hirsch, D.J., Song, S.Y., Dillahey, L.F., Williams, J.R., Snyder, S., 1998. Photodynamic phototherapy: mitochondrial benzodiazepine receptors as a therapeutic target. Mol. Med. 4, 40–45.

Weishaupt, K.R., Gomer, C.J., Dougherty, T.J., 1976. Identification of singlet oxygen as the cytotoxic agent in photoinactivation of a mouse tumour. Cancer Res. 36, 2326–2329.