Enhancement of photodynamic therapy in gastric cancer cells by removal of iron

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PAPERS

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Abstract

***Background*—Aminolaevulinic acid (ALA) is an endogenous substrate in the haem biosynthetic pathway. Protoporphyrin IX (PPIX), the immediate haem precursor in the pathway, has photoexcitable properties. Exogenous ALA has been used previously as a precursor agent in photodynamic therapy (PDT). Its main advantage is a short half-life and hence reduced incidence of skin photosensitivity. ALA can be toxic, however, causing, for example, transient increases in liver enzyme concentrations when given systemically and this may be dose related.**

***Aim*—To assess whether accumulation of PPIX and ultimately the efficacy of PDT could be improved by modulating both ends of the haem biosynthetic pathway.**

***Methods*—Gastric cancer cells (MKN 28) were incubated with ALA (0–1000 μmolar) and desferrioxamine (0–800 μmolar) for 24 hours before exposure to argon-pumped dye laser (630 nm) at different energy levels (0–40 J/cm^2). Cell viability was assessed by use of the methyl-tetrazolium (MTT) assay four hours after exposure to light.**

***Results*—Total PPIX accumulation increased linearly with increasing extracellular concentrations of ALA up to 1 mmolar (r=0·973, p<0·005). Adding 200 molar of desferrioxamine trebled PPIX accumulation over the same period of incubation. Cell viability after exposure to light decreased with low doses (0–30 μmolar) of desferrioxamine (r=–0·976, p=0·024). However, higher doses of desferrioxamine (more than 40 molar) seemed to confer a protective effect against PDT.**

***Conclusion*—PDT using ALA can be improved by removal of available iron with desferrioxamine. The reason for the protective effect of desferrioxamine seen at higher doses is not clear.**

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Keywords: photodynamic therapy; aminolaevulinic acid; ferrochelatase; desferrioxamine; gastric cancer cells; laser

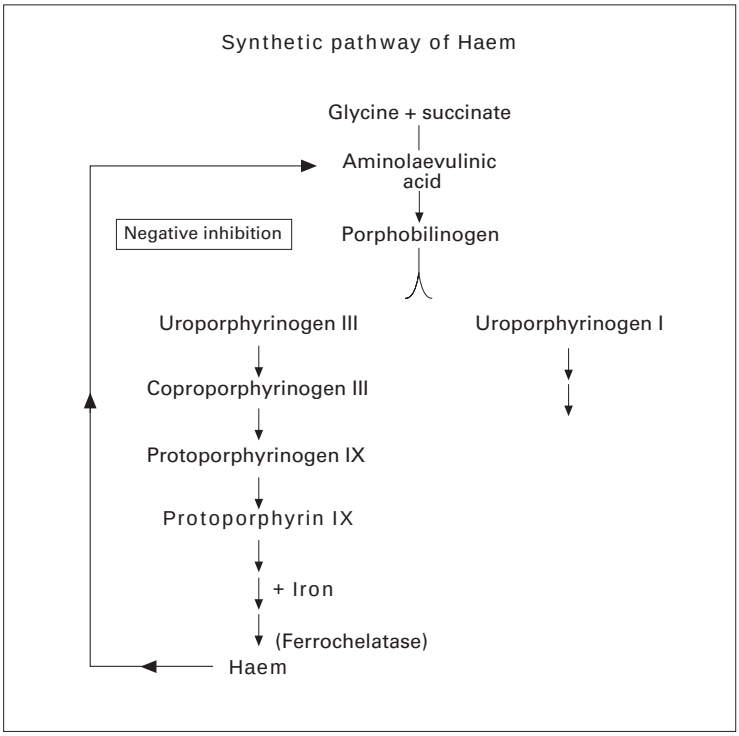

Figure 1: Synthetic pathway of haem. Protoprophyrin IX (PPIX) is the effective photosensitiser; its conversion to haem requires the enzyme ferrochelatase and iron. Removal of iron may inhibit ferrochelatase activity.

Photodynamic therapy (PDT) is a relatively new modality in the treatment of neoplasia. It involves pretreatment of a tissue with a photosensitiser which causes release of singlet oxygen upon exposure to light, resulting in cell destruction.[1] The photosensitisers most commonly used are haematoporphyrins and their derivatives.[2–4] Although effective as photosensitisers in PDT, the haematoporphyrins cause prolonged skin photosensitivity. Protoporphyrin IX (PPIX), a naturally occurring intermediate in the biosynthetic pathway for haem, has photoexcitable properties but is usually converted rapidly to haem in the presence of iron by the enzyme ferrochelatase (fig 1). Furthermore PPIX concentrations are kept low because haem has a negative feedback effect on aminolaevulinic acid (ALA) synthetase, the rate limiting enzyme in the haem biosynthetic pathway. This enzyme catalyses the conversion of glycine and succinyl coenzyme A to ALA. Exogenous administration of ALA can bypass this haem regulated step and results in a significant accumulation of PPIX in the cell.[5]

After administration of exogenous ALA, Bedwell *et al*[6] found that PPIX accumulation in tumour was six times that of normal mucosa.

A similar differential accumulation occurred between mucosa and submucosa. Thus, relative sparing of non-tumour tissue may occur if superficial mucosal lesions were treated in this way. The principle of using ALA to cause accumulation of PPIX results in destruction of superficial tissue after exposure to light[6] and has been exploited previously for superficial neoplastic or dysplastic lesions of the skin[7] and gastrointestinal tract.[8 9] PDT using ALA (ALA/PDT) has some potential, therefore, for superficial lesions involving a large surface area such as Barrett's metaplasia. Barr *et al*[10] treated five patients with Barrett's high grade dysplasia using ALA/PDT and the abnormal mucosa was subsequently replaced by squamous epithelium with eradication of dysplastic tissue. An additional advantage of using ALA as a photosensitiser is its short half-life so that skin photosensitivity and other adverse effects should be short-lived.

The purpose of this study was to determine whether the removal of iron from cells, thereby limiting its availability to ferrochelatase in the haem biosynthetic pathway, would result in increased accumulation of PPIX after administration of ALA. This could improve the efficacy or safety of ALA/PDT in gastro-oesophageal malignancy.

Methods

CELL CULTURE

Monolayer gastric cancer cells (MKN 28), derived from a well differentiated human tubular adenocarcinoma consisting of mucus secreting epithelial cells,[11] were used. This cell line was chosen based on the assumption that it may have properties unique to gastro-oesophageal adenocarcinoma which could be relevant to the treatment of Barrett's mucosa. The cells were cultured in RPMI (Gibco, Paisley, UK) culture medium supplemented with 10% (v/v) fetal calf serum (FCS), 100 units/ml penicillin and 100 μg/ml streptomycin. The cells were incubated in 75 cm^2 flasks (Costar, High Wycombe, UK) at 37°C with 5% carbon dioxide in humidified oxygen.

QUANTITATION OF PORPHYRIN

The ability of MKN 28 cells to accumulate PPIX in the presence of ALA was first confirmed by methods described previously.[12] Cells (10^6) were subcultured in 9·5 cm^2 six well tissue culture plates (Costar) with 2 ml FCS supplemented RPMI. The cells were incubated for 48 hours to about 80–90% confluence. The culture medium was then removed and the cells washed with 2 ml phenol red free Hank's balanced salt solution (HBSS). ALA (Sigma, Poole, Dorset, UK; 0–4000 μmolar) in phenol red free RPMI supplemented with 10% FCS was added to the cells to a final volume of 2 ml per well. The cells were incubated for another 24 hours before PPIX extraction. In experiments investigating the effects of iron status on PPIX accumulation, desferrioxamine (Sigma, 0–800 μmolar), an iron chelator, and ferric citrate (Sigma, 20 μmolar) were added in addition to ALA.

At the end of the incubation period the culture media were transferred to a plastic tube (GL 4) and centrifuged at 2000 revolutions for 10 minutes (Mistral 6L centrifuge). The supernatant (1 ml) was transferred to another tube and 1·5 ml 1% perchloric acid and methanol (1:1 v:v) was added to extract the porphyrins. Cells adhering to the wells were rinsed with 2 ml phenol red free HBSS and washed with 2·5 ml of the perchloric acid:methanol mixture for 10–15 minutes to extract PPIX. The extracts were then transferred to a plastic tube and centrifuged at 2000 revolutions (Mistral 6L) for 10 minutes to precipitate cell debris in the extracts and prevent it from interfering with PPIX measurement.

PPIX fluorescence was measured with a Perkin-Elmer 203 fluorescence spectrophotometer. The excitation wavelength was 405 nm with an emission wavelength of 605 nm. PPIX concentrations were calculated from a standard PPIX concentration curve. Protein was measured using Lowry's method.[13] The attached cells after PPIX extraction were digested with 1 ml 1 M sodium hydroxide at 60°C for one hour. The total amount of PPIX was expressed as the sum of PPIX extracted from the culture media and adherent cells in ng/mg cell protein.[12]

EXPOSURE TO LIGHT

In this study, 10^4 cells were subcultured in 96 well plates (Costar) with 200 μl FCS supplemented RPMI. After incubating for 48 hours, the cells were washed with phenol red free HBSS, and ALA (0–800 μmolar) and desferrioxamine (0–800 μmolar) were added. The cells were then incubated for a further 24 hours before being exposed to light.

The light source was an argon-pumped dye laser (Spectra-physics) emitting green light at 400 nm. The light was converted into red light (630 nm) by passing through kiton red dye and delivered through a microlens. The power density used varied between 8 and 10 mWatt/cm^2 and the total energy delivered at 0–40 J/cm^2. Wavelength of the light was verified by a Beck wavelength reversion spectroscope and the power confirmed by an integrating sphere. The temperature increase was confirmed to be less than 2°C by using a thermo-couple device during exposure to light.

The methyl-tetrazolium assay (MTT, Sigma) was used for the assessment of cell viability. This is a colorimetric assay which has been established as a reliable method of assessing cell viability in PDT studies.[14] Cell viability was assessed at four hours after exposure to light when it was at its trough (fig 2). MTT, 20 μl dissolved in phosphate buffered saline (5 mg MTT/ml phosphate buffered saline, Gibco), was added to the cells. After four hours' incubation at 37°C, culture medium containing MTT was removed, 100 μl dimethylsulphoxide (DMSO, Sigma) was added to solubilise the blue formazan formed by the viable cells. The plates were read by

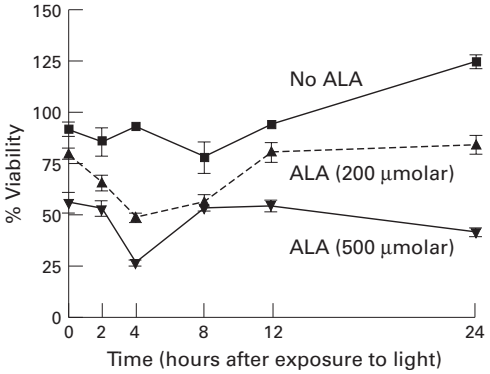

Figure 2: Time course of the MTT assay after exposure to light. Maximal cell death occurred at four hours after exposure to light. Light dose=40 J/cm².

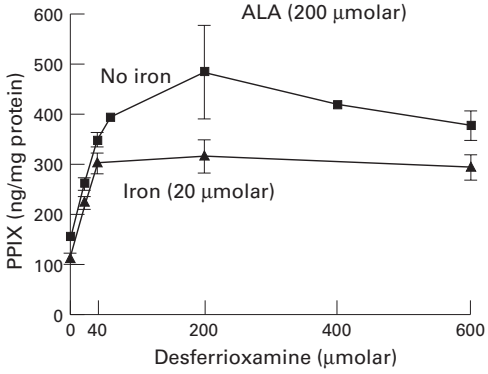

Figure 4: Accumulation of PPIX was increased by removal of iron using desferrioxamine. Adding iron cancelled this increase.

using an ELISA plate reader at 540 nm. Controls were cells exposed to a similar chemical environment but not light. Cell viability was expressed as a percentage ratio of exposed cells to control cells.

Results

ACCUMULATION OF PPIX

MKN 28 cells accumulated PPIX in the presence of ALA in a linear fashion up to 1000 μmolar ALA and concentrations seemed to plateau thereafter up to 4000 μmolar (fig 3). PPIX was characterised by its emission peak of 605 nm at 405 nm excitation wavelength. Detection of the second peak of PPIX at about 650 nm was not possible with our spectrophotometer but the band was confirmed to be identical to a PPIX standard (purchased from Sigma) by HPLC (courtesy of the Department of Biochemistry, Hope Hospital, Salford, UK).

EFFECT OF DESFERRIOXAMINE

Desferrioxamine had a dose dependent negative effect on cell viability in the absence of light (dark toxicity) up to 200 μmolar presumably because of removal of iron required for growth. Addition of iron or ALA did not cause dark toxicity. The dark toxicity of desferrioxamine should not influence the result of ALA/PDT in this study as the cell viability rate was expressed as a percentage of cells exposed to a similar chemical environment but not to light.

Addition of desferrioxamine to the cell culture in the presence of ALA increased accumulation of PPIX (fig 4). PPIX accumulation in the presence of 200 μmolar desferrioxamine was up to three times that without desferrioxamine. The effect was diminished when iron was added, suggesting that the effect of desferrioxamine was related to iron chelation. PPIX did not accumulate with the addition of iron or desferrioxamine to cells in the absence of exogenous ALA.

RELATION BETWEEN CELL VIABILITY AND LIGHT ENERGY LEVELS

Cell viability decreased with increasing energy levels (fig 5). Addition of ALA to the media increased the cell kill rate on exposure to light in a dose related manner. At 40 J/cm² light energy, 50% of cells were killed with 500 μmolar ALA. Cell viability dropped to 20% with 800 μmolar ALA. However, a comparable cell survival curve was achieved by adding 40 μmolar desferrioxamine in the presence of 500 μmolar ALA.

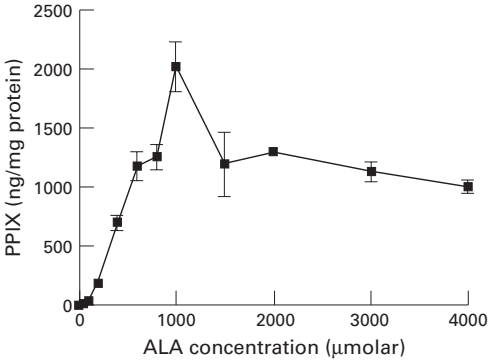

Figure 3: Accumulation of PPIX in relation to ALA concentration. There is a linear correlation between PPIX accumulation and ALA concentrations up to 1 μmolar of ALA. PPIX accumulation fell but plateaued between 1 and 4 μmolar.

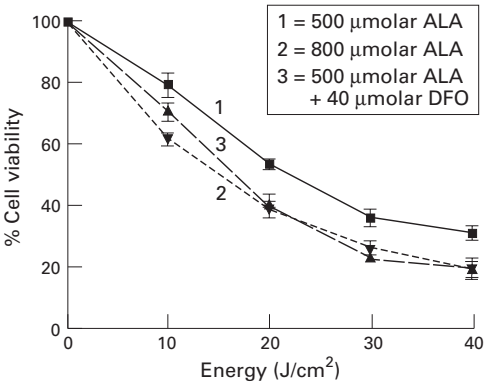

Figure 5: Cell viability in relation to energy levels. Line 1, cells given 500 μmolar ALA alone; line 2, cells given 800 μmolar ALA; line 3, cells given 500 μmolar ALA and 40 μmolar desferrioxamine (DFO). Adding desferrioxamine improved the efficacy of photodynamic therapy.

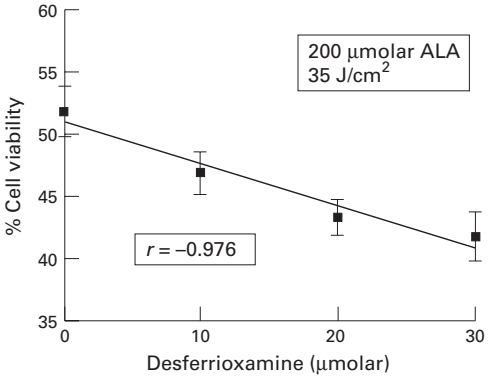

Figure 6: Negative correlation between cell viability and desferrioxamine concentrations up to 30 μmolar. Light dose=35 J/cm², 200 μmolar ALA.

Without exogenous ALA, 20, 200 and 800 μmolar desferrioxamine alone did not cause effective PDT with cell viability at 95, 92 and 98% respectively after exposure to light at 35 J/cm². There was a negative linear correlation (r=–0·976, p=0·024) between desferrioxamine and cell viability up to 30 μmolar in the presence of ALA (fig 6). The effect of desferrioxamine on cell viability after exposure to light seemed to reverse after 40–60 μmolar desferrioxamine – that is, there was a 'J' shape relation so that at higher doses of desferrioxamine, it seemed to confer a protective effect on cells against laser light (fig 7). We were unable to demonstrate a free radical scavenging effect of higher dose desferrioxamine using a thiobarbituric acid (TBA) assay or singlet oxygen quenching using rate of triplet oxygen decay to explain the J shaped survival curve.

Discussion

The use of PDT in the treatment of gastrointestinal malignancy has attracted much interest.[4 8 15–17] Haematoporphyrins and their more pure derivative photofrins have been the major photosensitisers studied.[4 16–18] Complete eradication of superficial tumours with minimal adverse effects has been reported.[4 18] However, these "conventional" photosensitisers caused prolonged skin phototoxicity and visceral strictures.[4 18] The latter may be owing to lack of selectivity of the photosensitisers in tissue distribution causing collagen damage in the muscularis and muscularis propria which then heals by fibrosis.[4 10 18] ALA may permit a differential accumulation of PPIX in the mucosal layers of the gastrointestinal tract[6 19] conferring relative selectivity of PDT for mucosal neoplasia. In clinical series reporting use of ALA/PDT for gastrointestinal neoplasia, superficial necrosis of the tumour mucosa has been demonstrated and the main adverse effects were transient skin phototoxicity and a rise in liver enzyme concentrations.[8 9] In our clinical studies of ALA/PDT (unpublished data), patients given higher doses of ALA (75 mg/kg *v* 60 mg/kg) achieved higher concentration of PPIX in the tissues as measured by fluorescence microscopy. However, higher doses and repeated exposure to ALA was associated with more noticeable and frequent increases in liver enzyme concentrations, limiting the use of ALA at higher doses.

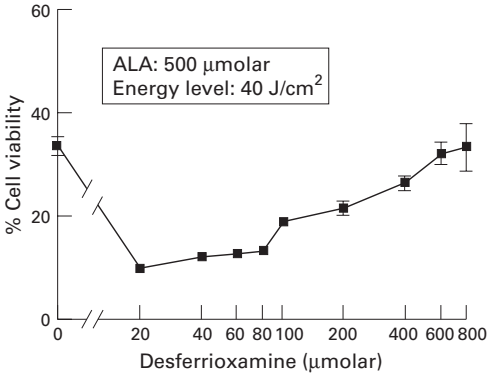

Figure 7: Cell viability in relation to desferrioxamine concentrations. There was a 'J' curve effect at higher doses of desferrioxamine, possibly because of its antioxidant properties.

This study has shown that removal of available iron from a gastric cancer cell line using desferrioxamine in combination with ALA results in increased PPIX accumulation, which in turn increased the efficacy of PDT. Although desferrioxamine is not a specific chelator of iron, the addition of iron abrogated the effect, supporting the contention that the removal of iron had an important role and this is certainly a plausible theoretical explanation. In addition, a study by He *et al*[20] on epidermal keratinocytes and epidermoid skin cancer cells using EDTA as the chelator showed that removal of iron played an important part in decreasing the activity of ferrochelatase, leading to increased accumulation of PPIX. The present study also showed that manipulating the availability of iron in this metabolic pathway could increase cell killing owing to PDT by 30%. This observation has potential to be exploited clinically.

The main advantage of PDT over thermal laser ablation is the relatively selective destruction of neoplastic over normal tissue and that healing occurs by regeneration rather than scarring.[21] The selective destruction is achieved by differential distribution of photosensitisers. However, the selectivity of photosensitiser distribution is not absolute, especially with haematoporphyrin derivatives.[10] This may result in damage to surrounding tissues and stricturing. Even with ALA induced photosensitisation, the differential PPIX accumulation within tissue in vivo[8] was not as high as in the ex vivo study,[6] particularly in the colon and at a lower dose of ALA (30 mg/kg).

Malignant cells may have special requirements for iron[22] making them more susceptible to the effect of iron chelation. Examining a variety of cell lines, Iinuma *et al*[23] also noted that desferrioxamine enhanced the accumulation of PPIX in carcinoma cells but not in non-malignant cell lines. High doses of desferrioxamine caused dark toxicity, possibly by complete inhibition of ferrochelatase activity, thereby depriving the cells of vital enzymes such as cytochrome or by removing iron from ribosomal reductase and impeding DNA syn-

thesis.[24] This property has been explored in relation to the treatment of hepatocellular carcinoma in vitro and in an animal model[25] using desferrioxamine in doses up to 120 μmolar.

Ferrochelatase in non-malignant cells may also be relatively more efficient so that more of the accumulated PPIX is converted to haem.[26] None the less, metabolic manipulation of the haem biosynthetic pathway, for example, with desferrioxamine, is likely to affect other tissues and although it may be reasonable to assume that the effect is relative, the extent of selectivity for malignant cells in vivo remains unknown. There are many other factors involved in the determination of PDT outcome, particularly vascular changes.[27] Further studies based on animal models will be required to assess PPIX distribution and the tissue response to PDT.

An intriguing observation in this study was that although cell viability on exposure to ALA and light was reduced with addition of desferrioxamine at low doses, it actually recovered again with higher doses, simulating a 'J' shaped viability curve (fig 7). Although generation of singlet oxygen is the more widely accepted explanation for the mechanism of cytotoxicity with PDT, generation and propagation of free radicals has also been postulated as another possible explanation for the cell damage.[28 29] The paradoxical effect of desferrioxamine at high doses may be related to its antioxidant activity at higher doses.[30] We were unable to find evidence of either effect using thiobarbituric acid to detect free radicals[30] or triplet oxygen decay as a measure of singlet oxygen quenching.[31] This phenomenon will require further study before manipulation of the haem pathway can be undertaken in vivo.

In conclusion, inhibition of ferrochelatase using desferrioxamine in the presence of ALA increased accumulation of PPIX; this, in turn improved the efficacy of ALA/PDT. However, the enhanced effect of ALA/PDT by desferrioxamine seemed to be dose related. At higher doses of desferrioxamine, there was a paradoxical decrease in cell phototoxicity. The explanation for this observation was not due to the anti-oxidation or singlet oxygen trapping property of desferrioxamine at higher doses. Clinical application of the observation using desferrioxamine to enhance ALA/PDT may be possible but will require careful titration of ALA dose in relation to desferrioxamine. Further studies are required.

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