Manuscript Title:

The effects of protoporphyrin IX-induced photodynamic therapy with and without iron chelation on human squamous carcinoma cells cultured under normoxic, hypoxic and hyperoxic conditions.

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Summary

Background

Photodynamic therapy requires the combined interaction of a photosensitiser, light and oxygen to ablate target tissue. In this study we examined the effect of iron chelation and oxygen environment manipulation on the accumulation of the clinically useful photosensitiser protoporphyrin IX (PpIX) within human squamous epithelial carcinoma cells and the subsequent ablation of these cells on irradiation.

Methods

Cells were incubated at concentrations of 5%, 20% or 40% oxygen for 24 h prior to and for 3 h following the administration of the PpIX precursors aminolevulinic acid (ALA), methyl aminolevulinate (MAL) or hexylaminolevulinate (HAL) with or without the iron chelator 1,2-diethyl-3-hydroxypyridin-4-one hydrochloride (CP94). PpIX accumulation was monitored using a fluorescence plate reader, cells were irradiated with 37 J/cm² red light and cell viability measured using the neutral red uptake assay.

Results

Manipulation of the oxygen environment and/or co-administration of CP94 with PpIX precursors resulted in significant changes in both PpIX accumulation and photobleaching. Incubation with 5% or 40% oxygen produced the greatest levels of PpIX and photobleaching in cells incubated with ALA/MAL. Incorporation of CP94 also resulted in significant decreases in cell viability following administration of ALA/MAL/HAL, with oxygen concentration predominantly having a significant effect in cells incubated with HAL.

Conclusions

Experimentation with human squamous epithelial carcinoma cells has indicated that the iron chelator CP94 significantly increased PpIX accumulation induced by each PpIX congener investigated (ALA/MAL/HAL) at all oxygen concentrations employed (5%/20%/40%) resulting in increased levels of photobleaching and reduced cell viability on irradiation. Further detailed investigation of the complex relationship of PDT cytotoxicity at various oxygen concentrations is required. It is therefore concluded that iron chelation with CP94 is a simple protocol modification with which it may be much easier to enhance clinical PDT efficacy than the complex and less well understood process of oxygen manipulation.

Keywords

Photodynamic therapy, oxygen manipulation, iron chelation, CP94, Protoporphyrin IX

Introduction

Photodynamic therapy (PDT) requires the combined interaction of a photosensitiser, light and molecular oxygen to ablate target tissue. Despite PDT being an established treatment modality within the field of dermatology [1] it has yet to become a widely used therapy. This may partly be due to limitations of current PDT regimens, for example, in achieving selective and sufficient accumulation of photosensitiser in the target tissue, optimising the light dosimetry, successfully delivering the light to the target areas and finally resolving the issue of the limited availability of molecular oxygen in the target tissue [2].

Oxygen is consumed in the photochemical reactions required for successful photodynamic therapy [3]. The photosensitiser in its excited triplet state can react with ground state molecular oxygen to undergo the type II photochemical reaction in which oxidised products and/or singlet oxygen are formed [4]. The oxidised products can then undergo type I photochemical reactions producing other cytotoxic species which along with singlet oxygen ablate tissue. Tissue destruction can arise from direct cytotoxicity, vascular damage, and through stimulation of host immune responses [4]. However, oxygen can be consumed within these photochemical reactions more rapidly than it can be replenished [5] and therefore can be a rate-limiting parameter. In addition, PDT can induce vascular shutdown, decreasing blood flow in both normal and tumour vessels, which results in hypoxia [6]. Hypoxia can reduce the PDT effect as well as stabilising the hypoxia-inducible factor-1 α protein (HIF-1 α) which increases vascular endothelial growth factor (VEGF) expression. The increased production and secretion of VEGF is thought to contribute to tumour

survival and regrowth via angiogenesis, glycolysis, erythropoiesis and apoptotic inhibition [7]. Finally, the cellular microenvironment of tumours tends to be hypoxic relative to normal tissue, which may influence protoporphyrin IX (PpIX) production and thus compromise PDT efficacy. This factor has therefore, made the investigation of the effects of oxygen concentration on both PpIX production and subsequent cell viability following irradiation a priority in both previous [8] and future research.

Manipulating levels of molecular oxygen and/or being able to effectively replenish diminished levels of oxygen could be clinically useful for PDT and so research is taking place to try to optimise current PDT treatment regimes. Previous methods to potentially enhance the mechanism of action of PDT through oxygen manipulation have included the use of light dose fractionation [8], inhalation of carbogen or 100% normobaric oxygen (NBO) [9] or being subjected to hyperbaric oxygen (HBO) [9,10]. Other strategies to increase PDT sensitivity by modulating skin oxygen tension include hyperthermia, and perfluorocarbons [11].

This in vitro study investigated how differences in the oxygen environment, prior to and following the administration of the PpIX precursors aminolevulinic acid (ALA), methyl aminolevulinate (MAL) or hexyl aminolevulinate (HAL) with or without the iron chelator 1,2-diethyl-3-hydroxypyridin-4-one hydrochloride (CP94), affected the PpIX fluorescence produced within human squamous epithelial carcinoma cells. Additionally, the effect PpIX-induced PDT with iron chelation at different oxygen concentrations had on cell viability was also investigated.

Materials and Methods

Chemicals and cells

Unless otherwise stated all reagents and chemicals were supplied by Sigma-Aldrich Chemical Company (Poole, UK). The A431 (human squamous epithelial carcinoma) cells were purchased from the European Collection of Cell Cultures (ECACC, Wiltshire, UK). Under aseptic conditions in a class II laminar flow cabinet, cells were cultured in Eagle's minimum essential medium (EMEM) with 10% foetal calf serum (FCS, standardised to give an iron concentration between 450 and 600 $\mu\text{g}/100\text{ g}$), 2% (200 mM) L-glutamine (2 mM final concentration) and 2% penicillin (200 U mL^{-1} final concentration) and streptomycin solution (200 $\mu\text{g mL}^{-1}$ final concentration). 10 mM stock solutions of ALA/MAL/HAL were prepared in phosphate buffered saline (PBS), adjusted to physiological pH (pH 7.4) using NaOH (0.5 mM), filter sterilised (0.22 μm Millipore) and stored at $-20\text{ }^{\circ}\text{C}$ for up to one month. Cells were grown in 5% CO_2 , 20% O_2 , at $37\text{ }^{\circ}\text{C}$ and left to grow until 70% confluent at which time cells were routinely passaged (every 3-5 days).

Oxygen manipulation

A431 cells were seeded into 2 x 96-well plates (Corning, NY, USA) at a density of 1×10^5 cells per mL (10^4 cells per well) and left to adhere in 5% CO_2 , 20% O_2 , at $37\text{ }^{\circ}\text{C}$ for 24 h. Following this, cells were incubated for a further 24 h in 5% CO_2 , at $37\text{ }^{\circ}\text{C}$ and in 5, 20 or 40% O_2 . The desired oxygen environment was achieved using a Tri-gas incubator (MCO-18M, Sanyo, San Diego, USA). After the elapsed 24 h incubation all medium was aspirated from the wells of both plates and 100 μL of appropriate test solution; modified EMEM + ALA/MAL/HAL $\pm$ CP94 (ALA, 250 μM ; MAL, 1000 μM ; HAL, 10 μM ; CP94, 150 μM) was added to each well (5 wells

per test solution), under dark room conditions. These doses were chosen based on results from previous work within the group which showed at the doses employed maximum PpIX fluorescence with minimal dark toxicity could be achieved [12]. Both plates were incubated for a further 3 h to allow PpIX accumulation to occur.

PpIX fluorescence monitoring *in vitro*

Following the elapsed 3 h, the plates were removed from the incubator and the lids taped to minimise any change to the oxygen environment. Both plates were then read for 'pre' PpIX fluorescence levels (prior to light irradiation) using a fluorescence plate reader (Synergy HT; BIO-TEK, Germany). Measurements were taken from the bottom of the plate wells with a 400 ± 30 nm excitation filter and a 645 ± 40 nm emission filter in place. Following the 'pre' fluorescence measurements of the two plates, one plate was subjected to red light irradiation (Aktilite, Galderma, UK, 37 J/cm^2 , 635 ± 2 nm) whilst the other remained in the dark as the dark control. Finally, both plates were measured for 'post' PpIX fluorescence levels (following light irradiation).

Neutral red uptake (NRU) assay

Following 'post' PpIX fluorescence measurements the medium containing the test solutions was removed from the wells and cells were washed three times with PBS. A volume of 100 μl serum free medium per well containing neutral red ($40 \mu\text{g ml}^{-1}$) was then added, and the plate returned to the incubator for a further 2 h. Following this the neutral red medium was removed, the cells washed with 150 μl of PBS and neutral red destain solution (50% (v/v) 96% ethanol; 49% (v/v) deionised water; 1% (v/v) glacial acetic acid) was added to the wells. Finally, plates were rapidly shaken for a

minimum of 10 minutes and the level of neutral red uptake, which is directly proportional to the amount of live cells in the cell culture, was measured using the excitation and emission wavelengths 530 nm (30 nm bandwidth) and 645 nm (40 nm bandwidth), respectively [13].

Data analysis and statistics

For the 96-well test plates the first 5 wells contained control cells incubated with modified EMEM only (blank controls). The PpIX fluorescence measurements from these wells were used to remove natural cellular PpIX autofluorescence from all subsequent measurements made from the same plate. For the cell viability data collected, the viability in each test group was recorded and cell viability was displayed as a percentage of the untreated control group within each individual oxygen concentration. All data points in the figures represent mean values from experiments carried out in triplicate. Statistical significance between individual groups was determined using the Student's t-test and an ANOVA was employed when considering differences between data sets.

Results

Pre and post PpIX fluorescence

Following exposure to an oxygen concentration of 5%, 20% or 40% and incubation with ALA/MAL/HAL $\pm$ CP94, prior to light irradiation and following light irradiation 'pre' and 'post' PpIX fluorescence levels were recorded, respectively. As expected, the observed trend was a decrease in PpIX fluorescence following light irradiation (Fig. 1A-F) indicating that PpIX photobleaching had occurred during the light delivery. With all three pro-drugs significantly ($p < 0.05$) more PpIX fluorescence was observed pre-irradiation when CP94 was co-administered (Fig. 1A-F) at all three oxygen concentrations investigated. At each oxygen concentration the greatest levels of PpIX fluorescence prior to light irradiation were observed for the cells incubated with 1000 μ M MAL $\pm$ CP94 (in the range of 25-111 arbitrary units (a.u.); Fig. 1C-D), followed by cells incubated with 250 μ M ALA $\pm$ CP94 (in the range of 10-79 a.u.; Fig. 1A-B). The least amount of PpIX fluorescence prior to light irradiation was observed for the cells incubated with 10 μ M HAL $\pm$ CP94 (in the range of 5-61 a.u.; Fig. 1E-F). Comparison of the results produced by each pro-drugs administered indicated that greater levels of PpIX fluorescence were achieved when cells were incubated with 1000 μ M MAL $\pm$ CP94 compared to levels achieved when cells were incubated with 10 μ M HAL $\pm$ CP94 (ANOVA, $p < 0.05$). When levels of PpIX fluorescence between 1000 μ M MAL $\pm$ CP94 and 250 μ M ALA $\pm$ CP94, and between 250 μ M ALA $\pm$ CP94 and 10 μ M HAL $\pm$ CP94 were compared using ANOVA to detect any significant difference ($p < 0.05$) none was found ($p = 0.153$ and $p = 0.463$, respectively).

Following incubation with 250 μ M ALA/10 μ M HAL and subjection to 5%/20%/40% oxygen the observed trend for the level of 'pre' PpIX fluorescence achieved was in the order 5% > 20% > 40% (Fig. 1A and E). When either pro-drug was combined with CP94 and subjected to 5%/20%/40% oxygen (Fig. 1B and F), levels of 'pre' PpIX produced were increased significantly at all three oxygen concentrations tested ($p < 0.05$). Following incubation with MAL and subjection to 5%/20%/40% oxygen the observed trend for the level of 'pre' PpIX achieved was in the order 5% > 40% > 20% (Fig. 1C). When coincubated with CP94 levels of PpIX produced were again observed to increase at all three oxygen concentrations ($p < 0.05$), with the greatest amount of PpIX produced when cells were incubated with 1000 μ M MAL + CP94 at 40% oxygen (Fig. 1D).

Measurement of PpIX photobleaching

PpIX photobleaching occurs when the reactive oxygen species (ROS) produced during the PDT photochemical reactions permanently alter PpIX. A positive correlation between PpIX photobleaching and cellular damage has been previously reported [14-17] and it is therefore a good indicator of PDT effectiveness. The difference between the 'pre' and 'post' PpIX fluorescence readings can be attributed to PpIX photobleaching and so these measurements were quantified and compared as both the percentage of preirradiation PpIX levels bleached on irradiation as well as the absolute level of PpIX photobleaching in arbitrary units (Fig. 2). Although no significant difference was observed when the percentage of PpIX photobleached was compared between corresponding treatment groups (Fig. 2A), there appeared to be a trend of increased PpIX photobleaching when each pro-drug was coincubated with CP94 (Fig. 2B). Using a Student's t-test to compare each of the pro-drug

alone and pro-drug + CP94 data sets, revealed a significant increase in PpIX photobleaching for all three pro-drugs when coincubated with CP94 at any of the oxygen concentrations tested ($p < 0.05$); with the only exception being 1000 μM MAL + CP94 compared to 1000 μM MAL alone at 5% oxygen which did not reach significance at this level. When the levels of PpIX photobleaching between the three oxygen concentration data sets were compared using an ANOVA to detect any significant difference ($p > 0.05$) none was found.

Cell viability

Following irradiation, the viability of cells was assessed with the NRU assay. No significant difference in cell viability was observed between the different oxygen concentrations ($p > 0.05$) for cells incubated with 250 μM ALA or 1000 μM MAL alone (Fig. 3A and B). However when the cells were coincubated with 250 μM ALA + CP94 a significant decrease was observed at 20% oxygen compared to 40% (Fig. 3A). When the cells were coincubated with 1000 μM MAL + CP94 a significant decrease at 5% oxygen compared to 40% (Fig. 3B) was detected. For cells incubated with 10 μM HAL $\pm$ CP94 the observed trend was a decrease in cell viability in the order of 5% > 40% > 20% oxygen concentration (Fig. 3C). With and without iron chelation a significant difference in cell viability was detected between the 5 and 20% and between the 40 and 20% oxygen concentrations ($p < 0.05$) with significantly more cytotoxicity occurring with 20% oxygen than with 40% oxygen. Notably, following incubation of 10 μM HAL $\pm$ CP94 at 5% oxygen no significant change in cell viability was observed (Fig. 3C).

When comparing the percentage of cell viability observed at the same oxygen concentrations between prodrug alone and prodrug + CP94 significant differences were detected between 250 μ M ALA alone and 250 μ M ALA + CP94 at 20 and 40% oxygen ($p < 0.05$; Fig. 3A). In addition, a significant difference was detected between 10 μ M HAL alone and 10 μ M HAL + CP94 at 20% and 40% oxygen ($p < 0.05$; Fig. 3C). No significant difference in cell viability was observed between 1000 μ M MAL alone and 1000 μ M MAL + CP94 at any of the oxygen concentrations tested ($p > 0.05$; Fig. 3B).

Discussion

During PpIX-induced PDT the combined interaction of PpIX, light of a specific wavelength and oxygen are utilised to ablate tumour cells. Administration of the porphyrin precursors ALA/MAL/HAL increased the synthesis of the endogenously produced photosensitiser PpIX within tumour cells (Fig. 1), and following the excitation of PpIX by light, in the presence of molecular oxygen induced cell death (Fig. 3). Oxygen is known to be essential for successful PDT to occur [18] and due to its consumption within these photochemical reactions oxygen is a rate-limiting factor of the therapy. By employing iron chelators PpIX accumulation can be enhanced further (Fig. 1) and by manipulating the oxygen environment the effectiveness of PDT may be optimised. Therefore in this study, A431 human squamous epithelial carcinoma cells were incubated with PpIX precursors with and without iron chelation whilst being subjected to an oxygen environment of 5%, 20% or 40%. PpIX fluorescence measurements were taken prior to and following red light irradiation and subsequent cell death was also measured. It was noted that significantly greater PpIX fluorescence occurred preirradiation when the iron chelator, CP94 was administered (Fig. 1). Also that substantial photobleaching occurred in all the groups on irradiation (Fig. 2) and this was significantly greater in the iron chelator groups (except with MAL at 5% oxygen). Taken together this suggested that the addition of an iron chelator resulted in a significant increase in the level of PpIX synthesis. However, as fluorescence was only measured at an entire well level we are unable to ascertain how much of this PpIX remained within cells following synthesis.

It is widely accepted that tumour cells tend to be more hypoxic compared to normal cells and it has been suggested that hypoxic cells are less affected by porphyrins

and light [19], and as a result may not be affected as much by reducing fluence rate or light fractionation [20]. Interestingly Wyld et al. [21] observed an increase in PpIX production under hyperoxic conditions when cells were incubated at 21% oxygen and compared with hypoxic conditions (<5% oxygen), although they did not consider oxygen concentrations higher than this. More direct effects of hyperoxygenation have also been investigated. One study [9] demonstrated that hyperoxygenation could improve tumour response by enhancing direct and secondary tumour cell death. This group hypothesised that hyperoxygenation oxygenated preexisting hypoxic cells and compensated for oxygen depletion during PDT [9]. Nevertheless, an obvious disadvantage of HBO is the practical feasibility [11] as a custom-built chamber is required and the authors also highlighted that if a hypoxic region is too far away for oxygen diffusion to take effect then improvement may be limited [9].

In this study although the greatest level of pre irradiation PpIX fluorescence (Fig. 1), and as a result the greatest observed PpIX photobleaching (Fig. 2), was found when cells were incubated with MAL + CP94 under hyperoxic conditions (40%) analysis of the subsequent cell viability measurements (Fig. 3) following light irradiation did not find increased cell death when 40% oxygen was employed. Nevertheless cell viability was only assessed at one time point, likely only measuring immediate necrotic cell death, therefore to measure levels of apoptotic cell death assessment at longer time points following irradiation should be considered in future investigations. Alternatively, as it was not possible to measure oxygen concentration within the wells following pro-drug treatment it is possible that fluctuations in oxygen concentration occurred when the plates (albeit taped shut) were removed from the incubator for analysis. Although it is unlikely that this would have had a significant effect on PpIX

accumulation over the previous 3 h incubation period, it may have adversely effected the oxygen concentration during the period of irradiation and thus treatment efficacy.

When ALA and MAL were administered alone no significant differences in cellular viability were observed with the three different oxygen concentrations investigated (Fig. 3), however when CP94 was administered with ALA or HAL significantly more cell death occurred at 20% oxygen + CP94 than at 40% oxygen + CP94 (Fig. 3). Although unexpected this latter result may be due to oxygen dependent effects on the haem biosynthesis pathway.

These preliminary in vitro findings suggest current dermatological PpIX-induced PDT regimens, carried out under normal atmospheric oxygen (~21%) conditions do not have a detrimental effect on efficacy but with further investigation the efficiency of PDT might be improved by employing iron chelation and maintaining sufficient oxygen availability during PpIX-induced PDT treatment. In this study iron chelation significantly increased PpIX photobleaching which corresponded to the increased accumulation of PpIX detected pre irradiation with the addition of CP94 to each congener and supports the findings of previous work [22-25]. In addition, these in vitro results suggest at 20% oxygen the use of HAL, which was used at much lower concentrations than either ALA or MAL, should also be considered for topical PDT. It has been demonstrated previously that with increasing concentrations of ALA a rise to plateau in PpIX fluorescence was observed [22] and previous work from our group have shown this to be when the concentration of ALA increases above 250 μ M [26]. In addition, above 10 μ M HAL dark cytotoxicity has been observed [12] and so much smaller concentrations of this ester derivative are therefore employed. This is

supported by results from an in vivo study on human basal cell carcinomas which demonstrated for application times of up to 24 h, HAL was identical to ALA as a PpIX precursor with respect to PpIX fluorescence intensity, depth of penetration, and distribution within the basal cell carcinoma, but had the added advantage that much smaller concentrations of HAL could be used [27]. In this study a significant decrease ($p < 0.05$) in cell viability was observed when HAL $\pm$ CP94 was incubated at 20% oxygen compared to either 5% or 40% oxygen suggesting greater cell ablation may be achieved in vivo.

It is important to remember, however, that in vitro results cannot be extrapolated into, for example, a dermatology clinic without supporting in vivo work because within multi-well plates effects are taking place within a closed system whilst in vivo there are many factors to be taken into consideration. For example, the heterogeneity of oxygenation due to micro- environmental differences in inter-capillary distance and pre-existing hypoxia which vary depending on the target tissue [28]. In addition, PDT can itself induce hypoxia but to date the current understanding of the role of hypoxia pre-, during- and post-PDT is limited. One group studied the relationship of tumour hypoxia and response to PDT in an experimental mouse model (radiationinduced fibrosarcoma tumour (RIF)) [19]. They concluded that considering the reduced effectiveness of photodynamic cell ablation at low oxygen concentrations, the rapid induction of tumour hypoxia by PDT itself, and the high tumour cure rate (81%) observed in their study, that in the RIF tumour, hypoxic tumour cells are inactivated by a mechanism other than direct photodynamic cytotoxicity [19]. This could be explained by the suggestion that hypoxia can induce necrosis via ATP depletion within cells [29]; implying PDT efficacy may be enhanced if hypoxia is induced by

light irradiation. On the other hand, hypoxia has been shown to stabilise HIF-1 α which in turn increases VEGF expression, with the increased production and secretion of the latter thought to contribute to tumour survival and regrowth via the promotion of angiogenesis, glycolysis, erythropoiesis and apoptotic inhibition [7]. In conclusion, the hypothesis that the efficiency of PpIX-induced PDT might be improved by sustaining a replete oxygen environment during treatment, not only for supporting ROS and singlet oxygen consumption, but also for inhibiting angiogenesis requires further investigation. In particular, in vivo studies are required to further investigate the clinical use of PDT with oxygen manipulation.

In conclusion, experimentation with human squamous epithelial carcinoma cells has indicated that the iron chelator CP94 significantly increased PpIX accumulation induced by each PpIX congener investigated (ALA/MAL/HAL) at all oxygen concentrations employed (5%/20%/40%) resulting in increased levels of photobleaching and reduced cell viability on irradiation. Further detailed investigation of the complex relationship of PDT cytotoxicity at various oxygen concentrations is required. It is therefore concluded that iron chelation with CP94 is a simple protocol modification with which it may be much easier to enhance clinical PDT efficacy than the complex and less well understood process of oxygen manipulation.

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Figure 1 Levels of PpIX fluorescence measured prior to (pre) and following (post) light irradiation of human squamous epithelial carcinoma (A431) cells subjected to 5%, 20% or 40% oxygen and incubated with: (A) 250 μ M ALA, (B) 250 μ M ALA + CP94 (C) 1000 μ M MAL (D) 1000 μ M MAL + CP94, (E) 10 μ M HAL and (F) 10 μ M HAL + CP94. Data represents the mean of 5 wells $\pm$ 1 standard deviation, carried out in triplicate (n = 3). *Represents a significant accumulation of PpIX between cells incubated with ALA/MAL/HAL alone and cells incubated with ALA/MAL/HAL + CP94 (p < 0.05).

Figure 2 Bar-charts showing the percentage of preirradiation PpIX levels bleached following irradiation (A), and the absolute level of PpIX bleached in arbitrary units (B) in human squamous epithelial carcinoma (A431) cells subjected to 5%, 20% or 40% oxygen and incubated with ALA/MAL/HAL $\pm$ CP94. Data represents the mean of 5 wells $\pm$ 1 standard deviation, carried out in triplicate (n = 3). *Represents a significant increase in PpIX photobleaching between cells incubated with ALA/MAL/HAL alone and cells incubated with ALA/MAL/HAL + CP94 (p < 0.05).

Figure 3 Cell viability of human squamous epithelial carcinoma (A431) cells (presented as a percentage of the blank control) following: (A) ALA $\pm$ CP94 PDT-treatment, (B) MAL $\pm$ CP94 PDT-treatment and (C) HAL $\pm$ CP94 PDT-treatment. Data represents the mean of 5 wells $\pm$ 1 standard deviation, carried out in triplicate (n = 3). *Represents a significant difference in cell viability (p < 0.05).

Figure 1

Figure 2

Figure 3