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Enhancement of methyl-aminolevulinate photodynamic therapy by iron chelation with CP94: an *in vitro* investigation and clinical dose-escalating safety study for the treatment of nodular basal cell carcinoma.

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Abstract

Purpose:

Methyl-aminolevulinate (MAL) photodynamic therapy (PDT) is a cancer therapy that combines the selective accumulation of a photosensitizer in tumor tissue with visible light (and tissue oxygen) to produce reactive oxygen species. This results in cellular damage and ablation of tumor tissue. Combining iron chelators with MAL has the potential to increase the accumulation of the photosensitizer protoporphyrin IX (PpIX) by reducing its bioconversion to heme. This paper investigates this method of enhancement both *in vitro* and for the first time clinically for the treatment of nodular basal cell carcinoma (BCC).

Methods:

Enhancement of MAL-induced PpIX accumulation by the iron chelator CP94 was quantified fluorometrically in human cultured cells (including three dermatological cell types). An open, dose-escalating, pilot study was then conducted in patients with nodular BCC, to determine the safety of this pharmacological modification.

Results:

Large enhancements in PpIX accumulation were observed in the cultured cells when co-incubated with the iron chelator CP94. Clinically the addition of CP94 was found to be feasible and safe. In addition greater reductions in tumor depth were observed in the CP94 co-incubated tumors.

Conclusion:

Iron chelation by CP94 is an effective enhancer of MAL-induced PpIX accumulation *in vitro*. This method of enhancement was safely applied to a clinical photodynamic therapy protocol with no unexpected adverse effects reported. Although the clinical investigation was only intended to be a small pilot to assess safety, enhancements in tumor clearance were observed both clinically and histologically when CP94 was included in the photosensitizing cream.

Keywords

Carcinoma, basal cell, 1,2-diethyl-3-hydroxypyridin-4-one (CP94), iron chelating agents, Metvix®, methyl-aminolevulinate (MAL), photochemotherapy.

Introduction

Photodynamic therapy (PDT) can be used to treat a wide variety of cancer types. The treatment combines three key components; a photosensitizer, visible light and tissue oxygen. When these are combined in tumor cells reactive oxygen species are produced. These highly reactive chemical species cause cellular damage and result in cell death (and ultimately the local ablation of tumor tissue) [1].

PDT has particularly found a niche in the treatment of dermatological tumors where light can be readily applied to the surface of the skin. Clinically substantial subsets of skin tumors are difficult to treat by conventional therapies (because of size, site or multiple lesion presentation). PDT can be advantageous in these situations and is also associated with excellent cosmesis making it a particularly attractive treatment option for cosmetically sensitive conspicuous sites [2].

Initially porphyrins such as haematoporphyrin derivative (HpD) were used as photosensitizing agents [3]. However their large molecular weight meant they had to be administered systemically. This combined with the prolonged photosensitivity associated with these compounds (up to three months) made this type of PDT less desirable. However in 1987 a novel and ingenious method of sensitizing tumors with porphyrins from a topically applied pro-drug was proposed [4]. This method involved administering an intermediate of the heme biosynthesis pathway (aminolevulinic acid (ALA)). ALA having a lower molecular weight than HpD is easily absorbed through the skin and enters cells where it acts as a substrate for the production of heme, bypassing the normal negative feedback inhibition controls on this enzyme pathway and resulting in large quantities of heme and heme precursors accumulating

in the cell. The intermediate preceding heme is protoporphyrin IX (PpIX) a porphyrin type photosensitizer. Therefore this method results in the photosensitization of cells. Advantages of this type of PDT are that photosensitivity by topical application can be contained at the site of application (and is not systemic as for HpD). In addition because PpIX is part of a natural enzyme pathway it is readily metabolized and removed from cells resulting in much reduced periods of photosensitivity (typically lasting only 24-48 hours).

ALA-PDT is now widely used in the treatment of dermatological tumors and the methyl ester of ALA (MAL; commercially Metvix®) has now gained regulatory approval in the UK and parts of Europe for the treatment of low risk basal cell carcinoma (BCC) and actinic keratosis (AK) where there has been considerable treatment success. For example in a recent review by Morton *et al.* [5] clearance rates for AK ranged from 71 to 100% indicating the suitability of ALA-PDT for this lesion type.

PDT clearance rates for BCC, although being slightly lower than those observed with the standard treatments (standard surgery or cryotherapy), are not statically significantly different from the clearance rates achieved with these most commonly applied BCC treatment modalities [6,7] (with an average clearance rate of 87% for superficial BCC [8]). Efficacy for the thicker nodular subset of BCCs however appears to be inferior to standard treatments unless prior de-bulking or repeated PDT treatments are performed [5,9]. Clearance rates for nodular BCC are much lower than those of superficial BCC with average clearances rates of just 53% [8]. Therefore thicker nodular tumors fare less well with PDT.

It is important therefore to try and improve the treatment of these thicker lesions in order to confer the benefits of PDT (better cosmesis, ability to treat large surface area/multiple lesions) to a wider number of patients. Various adaptations to the standard treatment have

therefore been considered. These include skin pre-treatment with the malignant cell differentiation potentiator dimethyl sulfoxide (DMSO) [10], the use of iron chelators [11], skin stripping with tape [12], light dose fractionation [13,14], low fluence rate light administration [15] as well as combinations with other techniques such as low dose Photofrin [16], hyperthermia [17,18], iontophoresis [19] and bioreductive drugs [20].

One of the most interesting of the methods above is the use of iron chelating agents. The mechanism of this technique is centered on to the heme biosynthesis pathway where PpIX is the immediate precursor of heme. The PpIX produced in the cell is converted into the non-photosensitizing compound heme by the enzyme ferrochelatase, which inserts iron into the tetra-pyrolle ring of PpIX. Binding the iron required for this reaction using an iron chelating agent should therefore reduce the amount of PpIX that is lost to heme, resulting in greater levels of photosensitizer temporarily accumulating in the cell (Fig 1).

Several authors have now investigated this technique of enhancement *in vitro* using ethylenediamine tetraacetic acid (EDTA) [21,11], desferrioxamine (DFO) [21,22] and the novel hydroxypyridinone iron chelator CP94 [23,24]. The ability of EDTA and DFO or alternately, CP94 and DFO to enhance PpIX photosensitizer accumulations have been directly compared *in vitro* [21,24]. DFO was found to be superior to EDTA in this role [21] and CP94 was found to be superior to DFO for this purpose [24]. Therefore the likely order of efficacy of these chelating agents for the enhancement of PDT has been proposed as: CP94>DFO>EDTA (although CP94 and EDTA have not been directly compared) [24].

With this in mind CP94 is the iron chelator that has been investigated here as it has the greatest potential to be a clinically useful enhancer of ALA/MAL-PDT. CP94 (1,2-diethyl-3-hydroxypyridin-4-one hydrochloride) is a member of the hydroxypyridinone family of iron

chelators originally developed to supersede DFO in the treatment of iron overload. CP94 is particularly effective at chelating intracellular iron; having both a lower molecular weight and higher lipophilicity than either DFO or EDTA [25]. Unlike the other iron chelators previously investigated with ALA/MAL-PDT, CP94 (having a much lower molecular weight than either DFO or EDTA) is also much more suited to topical application which is a particular advantage for dermatological PDT.

The ability of CP94 to enhance ALA-induced PpIX fluorescence in animal models and produce greater tumor necrosis after light administration has previously been demonstrated [26,27]. In humans CP94 has been investigated on healthy skin explants using both ALA and MAL with CP94 being shown to increase PpIX accumulations in both cases [28]. However to date no clinical studies have been conducted investigating enhancement of MAL (Metvix®)-PDT by CP94 for the treatment of human skin cancers.

The feasibility of combining iron chelators to treat human skin tumors has been previously demonstrated by Fijan *et al.* [29] with the iron chelator DFO. This study included the treatment of 34 superficial BCCs and 22 nodular BCCs with ALA and the iron chelator DFO. No control groups were included however in this study so it is difficult to conclude what effects the inclusion of DFO had on tumor clearance compared to treatment with standard ALA-PDT.

Control groups were included by Liu *et al.* [30] and Choudry *et al.* [31] when investigating EDTA and DFO respectively in matched skin lesions. Lui *et al.* [30] used tumor depth as an endpoint and found a significant ($p < 0.01$) reduction in tumor depth in lesions treated with EDTA in combination with ALA. Choudry *et al.* [31] used surface fluorescence as an endpoint and concluded that there were no significant differences in surface fluorescence

between lesions co-incubated with and without DFO and ALA (the use of surface fluorescence would not have detected differences in PpIX fluorescence in the deeper parts of the lesions where the accumulation of PpIX is thought to be limited).

This investigation studies the effects of CP94 on MAL-induced PpIX fluorescence *in vitro* using human cultured cells of dermatological origin and *in vivo* clinically where tumor depth in nodular BCC following a Metvix®-PDT protocol was assessed. This is the first clinical investigation conducted into the feasibility of combining CP94 with Metvix® to enhance the efficacy of PDT for the treatment of nodular BCC.

Materials and methods

In vitro measurements of MAL-induced-PpIX enhancement by CP94

All media and disposable plastic equipment were purchased from Sigma (Poole, UK) unless otherwise stated. Human fetal lung fibroblasts (MRC-5), human skin fibroblasts (84BR) and human epidermal skin carcinoma cells (A431) were obtained from the European Collection of Cell Cultures (ECACC, Wiltshire, UK). Cells were maintained in Eagle's minimum essential medium (EMEM) supplemented with 10% (20% for 84BR cells) iron standardized fetal calf serum (standardized to give an iron concentration between 450-600 μg / 100 g), 2% (200 mM) L-glutamine and 2% (200 U/ml) penicillin and (200 μg/ml) streptomycin solution. Cells were grown in 5% CO_2 at 37 ˚C and passaged every 3-5 days as required. Normal human epidermal keratinocytes (NHEK) were obtained from PromoCell (Heidelberg, Germany) and cultured in a specifically designed medium - Keratinocyte Growth Medium 2 (PromoCell). Cells were grown in 5% CO_2 at 37 ˚C and passaged approximately every 5-7 days. All cell culture work was carried out using aseptic technique in a Class II biological safety cabinet.

The MRC-5 cells were chosen because they are fast-growing, reliable and well characterized primary cultured cells. The other three cell types were all dermatological in origin. The 84BR and NHEK cells were selected to represent normal human skin cells that may be encountered during dermatological MAL-PDT, while the A431 cells were chosen to represent human epidermal tumor cells.

Before conducting PpIX fluorescence measurements, cell viability was assessed using the trypan blue exclusion method and was > 98% for all experiments. Prior to experimentation

cells were seeded into 96-well plates (Corning, flat bottom, cell culture treated) at a density of 10^5 cells per ml (10^4 cells per well) and incubated overnight at 37 ˚C in 5% CO_2. At these densities the cells had reached confluence after overnight incubation.

The following day all medium was removed and the cells washed with phosphate-buffered saline. All test solutions were freshly prepared before each experiment by dissolving MAL and CP94 into modified EMEM (minus phenol red). The pH of the solutions were checked and adjusted to physiological pH (pH 7.4) using NaOH (0.5 M) as necessary. Solutions were then filter sterilized (0.22 µm, Millipore filter) before being diluted to the final concentrations used. CP94 (synthesized as a powder) was kindly provided by Professor Hider (Kings College London, UK).

After the addition of the test solutions all procedures were carried out under reduced light levels (luminance in the laboratory was reduced to 50 lux) to reduce photo-bleaching of metabolized PpIX. PpIX fluorescence was measured using a fluorescence plate reader (Synergy HT, BIO-TEK, Germany). Measurements were made after an incubation period of 3 hours (for Figs 2 and 4) or 0-6 hours (for Fig 3). During these times cells were incubated in the dark in a 5% CO_2 incubator at 37 ˚C. The plate reader was pre-heated to 37 ˚C to reduce temperature-edge-effects. Measurements were taken with a 400 ± 30 nm excitation filter and a 645 ± 40 nm emission filter in place (from the bottom of the plate wells).

Each test plate contained control cells not incubated with any of the test solutions. These were used to remove background fluorescence from the data obtained (blanking). Each bar in the figures represents the mean of three readings. Statistical significance was determined using a one way ANOVA with a 95% confidence level.

Clinical pilot study of MAL-induced-PpIX enhancement by CP94

An open, dose-escalating, pilot study was conducted at the Royal Cornwall Hospital, Truro. Full ethical approval was obtained for the study and the Declaration of Helsinki protocols were followed. The patients were recruited from the standard outpatient clinic. Twelve patients participated in the study (4 males and 8 females), with a mean age of 79 years (range: 54-93 years). This number was chosen to establish the safety of this modified Metvix® formulation.

Each patient had a solitary, previously untreated, nodular BCC. Lesions were located on the back, face, neck or limbs. Each lesion had a minimum diameter of 5 mm and a maximum diameter of 25 mm. Patients under the age of 18 and pregnant women were excluded from the study. Patients within the child-bearing years were only included if they were able to use adequate contraceptive measures. Patients taking photoactive drugs were excluded. After written informed consent was obtained, a 3 mm punch biopsy was taken from the area within the lesion which appeared clinically to be the thickest. A 5.0 Vicryl rapide suture was used to close the biopsy site. Histology was performed on each specimen including Breslow thickness.

Three patients were treated in the control group (Metvix® alone) and 3 patients were treated with each escalating dose of CP94. Two weeks following the biopsy, the patients were allocated into one of the following groups in strict order of recruitment:

1) Metvix® alone (0% CP94)
2) Metvix® + 20% CP94

3) Metvix® + 30% CP94

4) Metvix® + 40% CP94

The lesions were digitally photographed and measured using skin landmarks to help isolate the exact area for future excision. Each lesion received one application of topical Metvix® (Galderma, UK) +/- the various doses of CP94 (depending on treatment group). The various concentrations of Metvix® with CP94 were made up in the pharmacy under strict protocol, no more than 1 hour prior to application to the lesion by simply mixing the required amount of CP94 powder into the Metvix® cream. No prior lesion preparation was performed and approximately 2 mm thickness of the cream was applied to the whole lesion and a 5 mm margin of surrounding normal skin, using a wooden spatula. The lesion was then covered with an occlusive dressing (Tegaderm® , 3M, Loughborough, UK) followed by a light occlusive Mepore® dressing as per the standard protocol for Metvix®-PDT. Patients were asked to comment on any adverse reactions such as irritation during the time of cream application and an observation of the treated area was made to assess any contact skin reaction such as erythema prior to irradiation.

Three hours following application of the cream, the lesions were illuminated with an Aktilite (Galderma, UK) LED light array (37 J/cm^2, 635 nm +/- 2 nm). This activating red light was delivered in a continuous manner to the entire lesion and to at least a 5 mm margin of adjacent skin. The treatment regime employed, apart from the incorporation of CP94, was therefore the same as our standard dermatological Metvix®-PDT protocol. In all cases, at 6 weeks following the single PDT treatment, clinical response rate was assessed and surgical excision of the whole treatment site was undertaken for histology. Previous photographs, skin

landmarks and precise measurements were used to ensure exact excision margins. Histological examination was carried out using thorough bread slice examination, including Breslow thickness estimation. Blood samples were taken for liver function tests prior to and 1 week following application of the iron chelator to assess any adverse effect on liver function.

Results

In vitro measurements of MAL-induced-PpIX enhancement by CP94

PpIX fluorescence was detected in the 500 µM only incubated cells after 3 hours incubation with a mean value of 47 arbitrary units (Fig 2). Co-incubation with the iron chelator CP94 (75, 150 and 300 µM) led to significant enhancements in the level of PpIX detected from the MAL only incubated MRC-5 cells ($p < 0.05$) (Fig 2). The increases in PpIX fluorescence from the MAL only treated cells increased as the concentration of CP94 was increased (by 0, 26, 29 and 31 arbitrary units respectively), although these increases were not statistically significant from each other (Fig 2). No significant levels of PpIX fluorescence were detected in the cells incubated with the iron chelator CP94 alone (without MAL) (data not shown).

When the level of PpIX fluorescence in the cells was measured over a 6 hour incubation period the level of PpIX detected in the MAL only treated cells was shown to increase over time (Fig 3). Co-incubation of MAL with CP94 (150 µM) was again shown to result in significant levels of PpIX enhancement at all time points ($p < 0.05$) (Fig 3).

Both the initial levels of MAL-induced PpIX and the degree of enhancement afforded by CP94 co-incubation were cell type dependent (Fig 4). The lowest initial levels of MAL-induced PpIX fluorescence were observed in the NHEK cells (24 arbitrary units). In these cells the smallest increase in PpIX fluorescence mediated by CP94 was also observed (3 arbitrary units) which was not statistically significant (Fig 4).

Similar initial levels of MAL-induced PpIX were detected in the 84BR cells and the A431 cells (~100 arbitrary units), these levels were much higher than those observed in the NHEK cells

(Fig 4). The enhancement mediated through the co-incubation of CP94 was however quite different in the 84BR and A431 cell types following the similar levels of MAL-induced PpIX initially observed. Co-incubation with CP94 in the 84BR cells led to a mean increase of 10 arbitrary units from the MAL only treated cells (this was not a statistically significant increase in PpIX fluorescence). In the A431 cells however a 50 arbitrary unit increase from ~100 arbitrary units was observed (which was statistically significant $p < 0.05$) (Fig 4). Therefore the greatest enhancement in MAL-induced PpIX fluorescence mediated by the iron chelator CP94 was observed in the skin tumor cell type (~50% increase).

Clinical pilot study of MAL-induced-PpIX enhancement by CP94

Twelve participants were included in the dose escalating clinical safety pilot study. Participants 1-3 received Metvix® + 0% CP94, participants 4-6 received Metvix® + 20% CP94, participants 7-9 received Metvix® + 30% CP94 and participants 10-12 received Metvix® + 40% CP94. No adverse effects above that normally experienced with Metvix®-PDT were reported from any of the participants in this study receiving any concentrations of CP94 with Metvix®. Liver function tests also remained normal following treatment.

All of the tumors from the participants treated with Metvix® alone had increased in depth 6 weeks following treatment. All of the patients treated with Metvix® + CP94 (20%, 30% and 40%) had reductions in initial tumor depth 6 weeks following treatment. Only one tumor was completely clinically resolved at 6 weeks, this tumor was in the Metvix® + 40% CP94 treated group (Fig 5).

Discussion

In vitro co-incubation of MAL with the iron chelator CP94 resulted in significantly ($p < 0.05$) enhanced levels of the photosensitizer PpIX being detected in the MRC-5 cells and the levels of enhancement observed increased slightly as the concentration of CP94 was increased (Fig 2). Significant enhancements in PpIX fluorescence were also detected at all time points when CP94 was co-incubated with 500 μM MAL for 0-6 hours in the MRC-5 cells (Fig 3). After only 1 hour the iron chelator CP94 was able to have an effect on the accumulation of PpIX and resulted in higher levels of this photosensitizer being detected in the cells, showing that both drugs readily enter cells and alter heme metabolism (Fig 3).

The MRC-5 cells above were chosen because they are a fast-growing, reliable and well characterized primary cultured cell type. The other cell types used in this study were selected to represent the types of cells that would be encountered in the skin during a normal PDT treatment session. The A431 cells were from an epidermal tumor (not BCC), while the NHEK and 84BR cells represented normal cells from the epidermis and dermis respectively. In the MAL treated skin tumor cells (A431) co-incubation with CP94 resulted in significant enhancements in PpIX fluorescence being observed (~50%); while in the normal skin cells investigated (84BR and NHEK) only minimal enhancements in PpIX fluorescence were seen (<15%). Differences in the initial level of PpIX fluorescence produced in the different cell types incubated with MAL alone were also observed (Fig 4).

Differential accumulation of PpIX in other cell types has previously been reported (neuroblastoma cells, hepatoma cells and fibroblast cells [32]) and in a study by Gibbs *et al.* [33] PpIX fluorescence was found to vary by as much as 10 fold amongst eight cancer cell

types investigated. Differing PpIX accumulation between normal and tumor human colonic cells has been found to be influenced by (but not solely determined by) differences in porphobilinogen deaminase (production of PpIX) and ferrochelatase (conversion of PpIX to haem) activities [34,35]. Iron metabolism has also been shown to be altered in tumor cells. In particular expression of the transferrin receptor has been shown to be up regulated in several tumor cells in response to an increased requirement for iron (which can influence heme biosynthesis).

As well as variations in initial PpIX levels reported above large variations have also been reported in the enhancement mediated by the addition of iron chelators. Only a 3-fold increase in PpIX fluorescence was observed in WiDr cells co-incubated with ALA and DFO while a 30-fold increase was observed with the same parameters in V79 cells [21]. Similarly large differences in enhancement were observed with CP94 in a study reported by Bech *et al.* [23] where 5 cell types were employed. Therefore PpIX fluorescence and the level of enhancement mediated through iron chelator co-incubation is cell type dependent. Although in all cell types some degree of enhancement in PpIX levels seems to occur when CP94 is employed.

The ability of CP94 to increase PpIX fluorescence particularly in tumor cells (A431) should be particularly advantageous in the treatment of deeper dermatological lesions or islands of cells where the concentration of MAL and MAL-derived PpIX may be already low. This would enable the production of clinically useful amounts of PpIX in tumor cells which would otherwise produce PpIX at a sub-lethal level. If this was to occur it would be expected that this would result in a better clinical outcome with greater reductions in tumor depth.

The *in vivo* part of this investigation was primarily designed to assess the clinical feasibility and safety of combining the iron chelator CP94 with Metvix® in a PDT protocol for the treatment of nodular BCC. This is the first clinical investigation of this simple pharmacological modification. No unexpected adverse events were noted for any of the 12 participants treated in this study and the clinical staff proceeded with the treatment as normal. The CP94 mixed well with the Metvix® cream and once mixed the modified Metvix® formulation was applied to the participants in exactly the same way as the standard Metvix®-PDT protocol. Therefore except for the preparation of the modified formulation by the pharmacist no additional inconvenience or time was required for this protocol modification. The formulation was prepared and used immediately. Investigation into the stability and shelf life of this modified formulation should be conducted in the future.

Although the main aim of this *in vivo* investigation was to determine the safety and feasibility of combining CP94 with Metvix®-PDT some interesting trends in efficacy were also observed. In the Metvix® only treated lesions (participants 1-3, Fig 5) no reductions in tumor depth were observed 6 weeks post treatment. Interestingly however, for all participants who received CP94 in the Metvix® formulation (participants 4-12, Fig 5), reductions in tumor depth were observed. In addition one of the CP94 co-treated BCC lesions treated in the study with the highest iron chelator dose (40%) completely resolved after 6 weeks.

This would not be expected from a standard Metvix®-PDT protocol with these thicker nodular BCCs, where prior de-bulking and two treatments 7 days apart would normally be require to achieve this result.

The small numbers of lesions treated in our investigation means that it is not possible to definitively conclude that CP94 enhances the treatment of nodular BCC lesions with Metvix

®-PDT. Never the less the observations from this study do suggest that combining CP94 with Metvix® is a safe and effective way of increasing the efficacy of Metvix®-PDT and further investigation of this phenomenon with a larger treatment group should now be conducted.

Similar reductions in tumor depth to those presented here have also been reported by Liu *et al.* [30] using the iron chelator EDTA combined with ALA-PDT for the treatment of non-melanoma skin cancer. Twelve patients were treated with ALA-PDT with and without the iron chelator EDTA in a paired design study. Tumor depth was determined using an Acuson Sequioa 512 phase-array system both before and 7 days after treatment. Significant reductions in tumor depth were observed in the EDTA co-treated lesions compared to the ALA alone treated lesions ($p < 0.01$) [30]. However again it should be noted that only a small number of lesions were treated in this study.

Despite these positive findings (above) in an *in vivo* investigation using the iron chelator DFO + ALA-PDT in matched skin lesions Choudry *et al.* [31] concluded that DFO iron chelation did not confer any additional benefits to standard ALA-PDT for non-melanoma skin cancer (although DFO + ALA was found to increase PpIX fluorescence in healthy skin). Only 8 patients with non-melanoma skin cancer took part in this study which investigated PpIX surface fluorescence from matched lesions. The large heterogeneity of PpIX accumulation in BCC tumors, the use of surface fluorescence (which would hot have determined any information from the deeper parts of the tumors) and the small number of patients included in this study may have masked the positive effects of iron chelation observed by ourselves and Liu *et al.* [30].

In addition the reported lack of enhancement in skin lesions using DFO + ALA may have been the result of differences between DFO and CP94. CP94 is of lower molecular

weight and higher lipophilicity than DFO. It is also neutrally charged in both iron free and iron complexed forms enabling it to move freely in and out of cells by simple diffusion [36]. CP94 may consequently enter intracellular iron pools more readily and this factor may account for the increased effect of CP94 over DFO.

The ability of CP94 to increase PpIX fluorescence particularly in cells where the concentration of MAL is low [24] may explain the increased effect in deeper nodular tumors. The addition of CP94 enabling the production of clinically useful amounts of PpIX deeper in the tumor, where PpIX production would otherwise be at sub-lethal levels.

The functions of iron in the cell are not limited to the haem biosynthesis pathway. Iron is needed for a number of important cellular functions including DNA synthesis (where it is required for the reduction of ribonucleotides to deoxyribonucleotides by the enzyme ribonucleotide reductase). Because of these other functions, removal of iron by chelation has previously been shown to result in antiproliferative effects via inhibition of DNA syntheses and it has been suggested that CP94 in particular may be able to more readily induce these effects due to faster access into intracellular iron pools [36]. This effect was not measured in this study but may be another way in which iron chelation may reduce tumor thickness in Metvix®/CP94-PDT. It should be noted that clinically CP94 is rapidly glucuronidated in humans and as such is quickly excreted from the circulation. Therefore any effects of CP94 on DNA synthesis clinically are likely to be transient.

Iron chelation by CP94 was found to be an effective enhancer of PpIX accumulation *in vitro*. This method of enhancement was then safety applied *in vivo* to a clinical photodynamic therapy protocol with no adverse effects reported. Although this study was only intended to be a small pilot to assess safety, enhancements in tumor clearance were observed when CP94 was

included in the formulation. From these initially promising findings it is recommended that a full clinical study now be undertaken to investigate this potentially beneficial method of treatment enhancement fully. The suitability of CP94 for topical application makes the use of a CP94/Metvix® formulation for the topical treatment of skin cancers a distinct possibility for the future, with the potential to benefit a large number of patients who currently only receive standard Metvix®-PDT.

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Figure legends

Fig 1. Simplified version of the heme biosynthesis pathway, indicating the effects of iron chelation on the conversion of PpIX to heme.

Fig 2. Stacked bar graph of mean PpIX fluorescence measured from MRC-5 cells incubated with 500 μM MAL and 0, 75, 150, or 300 μM CP94. Light gray bar denotes mean PpIX fluorescence from MAL only treated cells. Enhancement in PpIX fluorescence mediated by the iron chelator CP94 is shown by the dark gray bar.

Fig 3. Stacked bar graph of mean PpIX fluorescence measured from MRC-5 cells incubated with 500 μM MAL and 150 μM CP94 for 0, 1, 2, 3, 4, 5, or 6 hours. Light gray bar denotes mean PpIX fluorescence from MAL only treated cells. Enhancement in PpIX fluorescence mediated by the iron chelator CP94 is shown by the dark gray bar.

Fig 4. Stacked bar graph of mean PpIX fluorescence measured from normal human skin fibroblasts (84BR), normal human epidermal keratinocytes (NHEK) or epidermal carcinoma cells (A431) incubated with 500 μM MAL or 500 μM MAL+ 150 μM CP94. Light gray bar denotes mean PpIX fluorescence from MAL only treated cells. Enhancement in PpIX fluorescence mediated by the iron chelator CP94 is shown by the dark gray bar.

Fig 5. Results from dose-escalating clinical safety pilot study. For each individual participant initial histological tumor depth (mm) (yellow bar), calculated change in tumor depth (mm) (red bar) and histological tumor depth 6 weeks post treatment (mm) (green bar) are shown. Participants 1-3 received Metvix® + 0% CP94, participants 4-6 received Metvix® + 20% CP94, participants 7-9 received Metvix® + 30% CP94 and participants 10-12 received Metvix® + 40% CP94.

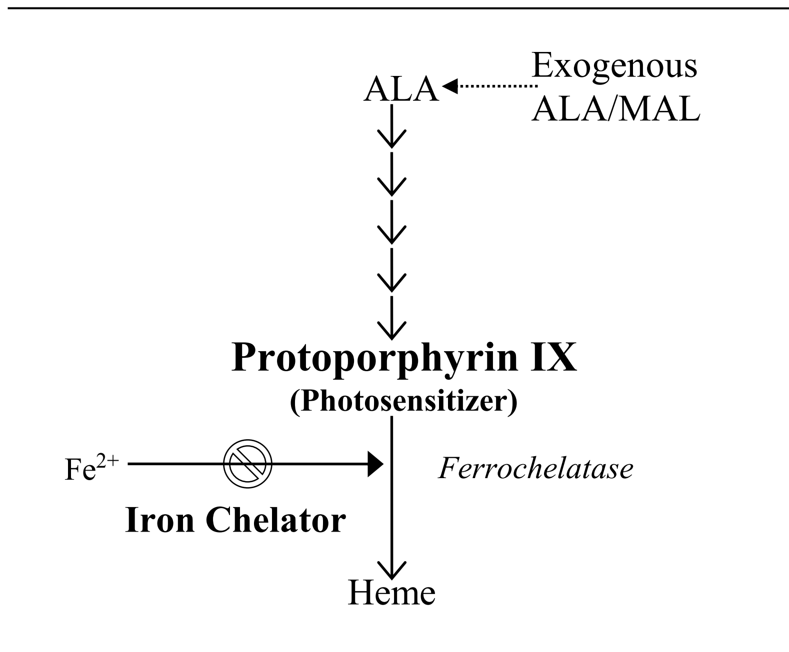

Figure 1

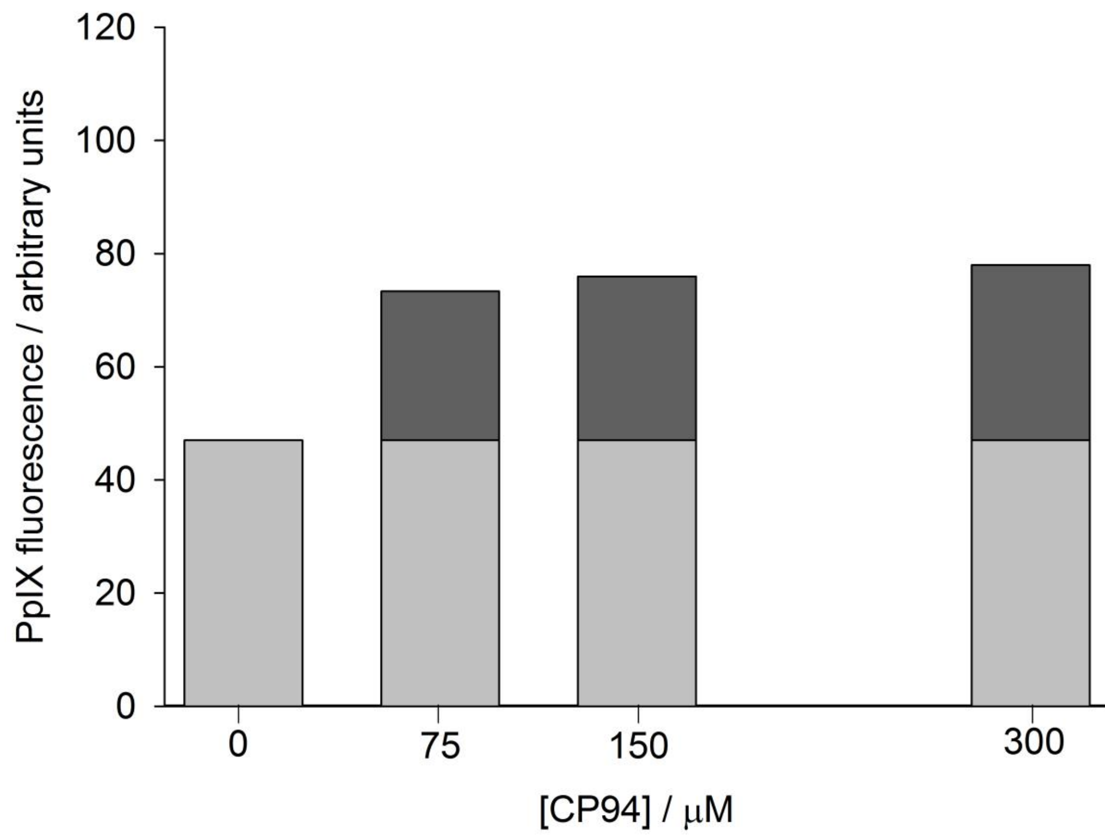

Figure 2

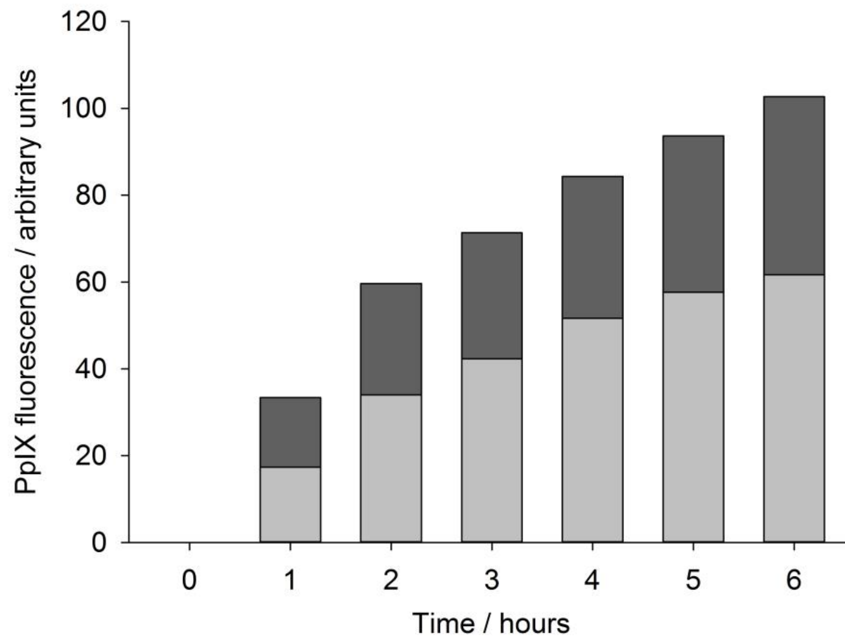

Figure 3

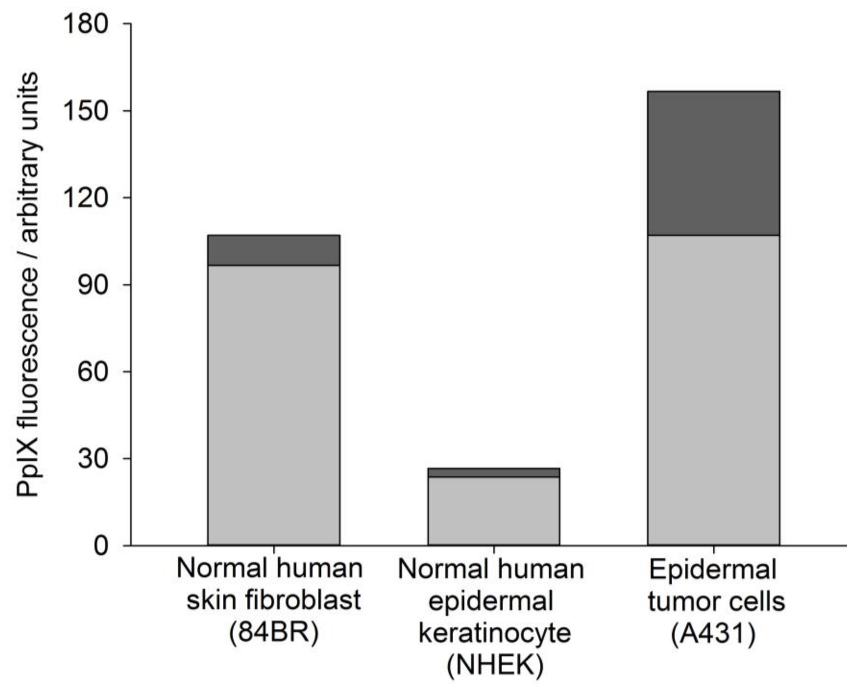

Figure 4

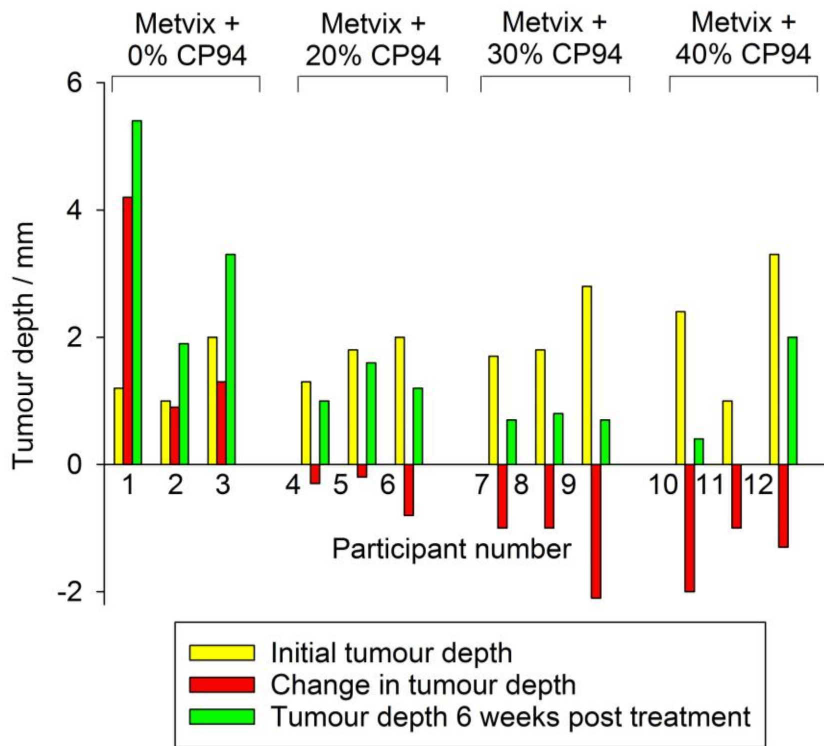

Figure 5