

## Increased efficacy of *in vitro* Photofrin<sup>®</sup> photosensitization of human oral squamous cell carcinoma by iron and ascorbate

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### Abstract

Photofrin<sup>®</sup>, a photosensitizer used in the photodynamic therapy of cancer, selectively localizes in cellular membranes. Upon exposure to visible light, Photofrin<sup>®</sup> produces singlet oxygen (<sup>1</sup>O<sub>2</sub>), which reacts with membrane polyunsaturated fatty acids forming lipid hydroperoxides. Transition metals, such as Fe<sup>2+</sup>, catalyze the production of cytotoxic free radicals from lipid hydroperoxides. Ascorbate reduces ferric to ferrous iron, further augmenting lipid peroxidation. Therefore, to increase the efficacy of Photofrin<sup>®</sup> photosensitization, we added 20 μM ferrous sulfate and 100 μM ascorbic acid, in an aqueous layer over SCC-25 oral squamous cell carcinoma cells during *in vitro* illumination. In electron paramagnetic resonance spin trapping experiments, using POBN (α-(4-pyridyl-1-oxide)-N-tert-butyl nitron), we observed that the presence of this pro-oxidant combination greatly increases the production of membrane-derived lipid free radicals. The effect was time dependent but only partially concentration dependent. Trypan blue dye exclusion demonstrated that this increase in lipid radical formation correlated with cytotoxicity. These observations support the hypothesis that Photofrin<sup>®</sup> photosensitization leads to lipid hydroperoxide formation, which increases the cell's susceptibility to iron-induced Fenton chemistry. The resulting free radical-mediated lipid peroxidation results in cell death. From these data we hypothesize that the efficacy of photodynamic therapy of superficial cancer might be increased by the topical application of the pro-oxidant combination of iron and ascorbate. Furthermore, their use will probably allow lower doses of Photofrin<sup>®</sup> without compromising antitumor effect. © 1997 Elsevier Science S.A.

**Keywords:** Photodynamic; Photofrin; Free radicals; EPR; Spin trapping

### 1. Introduction

Photofrin<sup>®</sup>, a partially purified preparation of hematoporphyrin derivative, is being used as a photosensitizer in the photodynamic therapy (PDT) of mucosal and non-mucosal squamous cell carcinoma [1–7]. The photodynamic action of Photofrin<sup>®</sup> relies upon the absorption of visible light to form its excited triplet state. This excitation energy is transferred to molecular oxygen, producing singlet oxygen, <sup>1</sup>O<sub>2</sub>. This highly reactive, electrophilic oxygen species initiates

oxidations that lead to cytotoxicity [8]. The hydrophobic character of Photofrin<sup>®</sup> causes it to localize in plasma and subcellular membranes, making these structures especially sensitive to the singlet oxygen produced upon photosensitization [9]. Singlet oxygen reacts with the carbon-carbon double bonds of polyunsaturated fatty acids producing lipid hydroperoxides (LOOH) [9]. We previously reported that the presence of Fe<sup>2+</sup> and ascorbate during Photofrin<sup>®</sup> photosensitization of L1210 leukemia cells increases the production of membrane derived lipid free radicals and enhances cytotoxicity [10]. The presence of ascorbate serves to reduce Fe<sup>3+</sup> to Fe<sup>2+</sup>, which readily donates an electron to LOOH, thereby initiating free radical chain reactions. Since the pro-oxidant combination of iron and ascorbate can enhance the cytotoxicity of PDT, we propose to manipulate their concentration to maximize PDT efficacy. However the delivery of iron and ascorbate, at high concentrations, to internal tumors may be difficult. Yet topical delivery to surface-oriented

Abbreviations: EPR, electron paramagnetic resonance spectroscopy; L<sub>d</sub>, Lipid-derived free radical; LOOH, lipid hydroperoxides; PDT, photodynamic therapy; POBN, (α-(4-pyridyl-1-oxide)-N-tert-butyl nitron); SCC, human squamous cell carcinoma.

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cancer, such as oral squamous cell carcinoma, is plausible. In this study we present *in vitro* data that suggests the *in vivo* efficacy of PDT with Photofrin® could be enhanced by applying iron and ascorbate topically.

## 2. Materials and methods

Photofrin® (porfimer sodium) was kindly provided by QLT Phototherapeutics, Inc., Vancouver, BC, Canada. It was suspended in dextrose 5% H<sub>2</sub>O (pH 7.4) and frozen until immediately before use. Ascorbate was prepared as a 0.10 M stock solution of L-ascorbic acid (Aldrich Chemical Co., Milwaukee, WI) in distilled H<sub>2</sub>O. Concentration was verified by dilution in metal-free phosphate buffer, pH 7.4, using  $\epsilon_{265} = 14\,500\text{ M}^{-1}\text{ cm}^{-1}$  for ascorbate [11].  $\alpha$ -(4-Pyridyl-1-oxide)-*N*-*tert*-butyl nitron (POBN) (OMRF Spin Trap Source, Oklahoma City, OK) was prepared as a 1.0 M stock solution in distilled H<sub>2</sub>O. Ferrous sulfate (Fisher Scientific, Fair Lawn, NJ) was prepared as a 20 mM stock solution in distilled H<sub>2</sub>O.

Human squamous cell carcinoma cells were obtained from ATCC (SCC-25 CRL 1628) and grown as a monolayer at 37°C (5% CO<sub>2</sub>) in medium consisting of a 1:1 mixture of Ham's F12 and Dulbecco's modified Eagles medium with 0.4  $\mu\text{g ml}^{-1}$  hydrocortisone, 10% fetal bovine serum and penicillin–streptomycin 100 units ml<sup>-1</sup>. Cells were grown to confluence in 60 mm tissue culture dishes at which time the

media were removed and replaced with 0.9% NaCl. The cells were then incubated for 45 min at 37°C in the dark with Photofrin® (15  $\mu\text{g ml}^{-1}$ ). At the termination of this incubation, the Photofrin®-containing saline was removed and replaced with 1.5 ml of fresh 0.9% NaCl. To the saline layer were added 100  $\mu\text{M}$  L-ascorbic acid, 20  $\mu\text{M}$  FeSO<sub>4</sub> and 25 mM POBN, in that order and in low ambient light conditions. Cells were exposed to visible light by placing the culture dish on a 115 V Picker fluorescent light source (X-ray viewing box in horizontal position). This delivered 48 J m<sup>-2</sup> s<sup>-1</sup> of light in the visible spectrum. Temperature was monitored and never exceeded 38°C. At designated time points 1.0 ml of the saline layer was removed and immediately placed in liquid nitrogen until analyzed by electron paramagnetic resonance spectroscopy (EPR). Cytotoxicity was determined by trypan blue dye exclusion. Briefly, the SCC-25 cells were manipulated as above in the absence of POBN. At designated time points the cells were removed from the plates with trypsin, diluted in fresh media and assayed for trypan blue dye exclusion.

EPR spin trapping analysis was performed using a Bruker ESP-300 EPR spectrometer. The frozen saline aliquots were thawed by placing them in a 37°C water bath, then immediately upon thawing placed in the EPR cavity in a TM EPR quartz flat cell. For the experiments in which the POBN radical adduct concentration was monitored versus time, each data point represents the signal-averaged result of five 20 s scans of the low field doublet of the POBN/lipid-derived

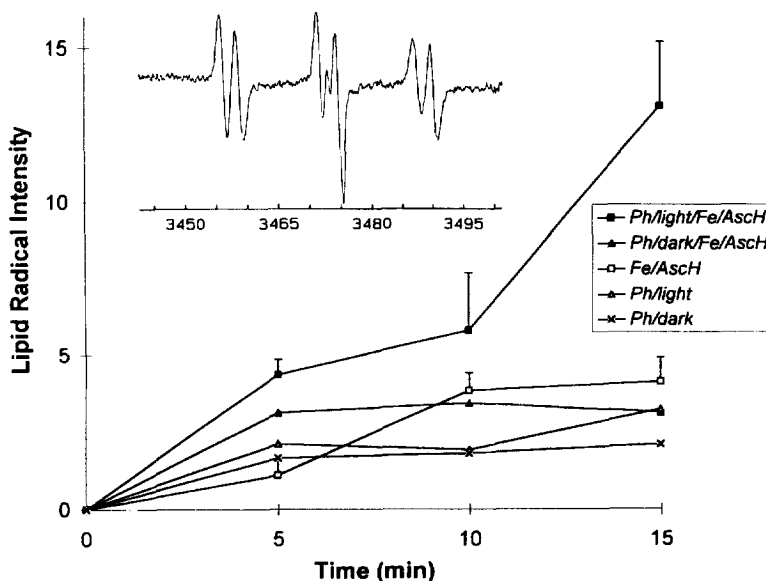


Fig. 1. The photodynamic action of Photofrin® on SCC-25 cells produces lipid radicals. Confluent SCC-25 cells in 60 mm tissue culture dishes were incubated with 15  $\mu\text{g ml}^{-1}$  of Photofrin® (in 0.9% NaCl) for 45 min at 37°C. At the termination of this incubation, the extracellular Photofrin® was removed and a thin layer of 0.9% NaCl containing 25 mM POBN with or without 20  $\mu\text{M}$  ferrous sulfate and 100  $\mu\text{M}$  ascorbic acid was placed over the cells. The cells were exposed to visible light by placing them on a 115 V Picker X-ray box. At designated time points, the thin aqueous layer containing the POBN was removed and immediately placed in liquid nitrogen until EPR analysis. The lipid radical intensity is in arbitrary units. INSET: EPR spectrum of the POBN/L<sub>1</sub> spin adduct from Photofrin® photosensitized SCC-25 cells. The spectrum consists of two radical species: the central doublet species ( $a^H = 1.8\text{ G}$ ) is the ascorbate free radical; the triplet of doublets is the POBN/L<sub>1</sub> spin adduct ( $a^N = 15.65\text{ G}$ ,  $a^H = 2.71\text{ G}$ ). This spectrum was obtained after 15 min of Photofrin® (15  $\mu\text{g ml}^{-1}$ ) photosensitization of SCC-25 cells in the presence of 20  $\mu\text{M}$  ferrous sulfate and 100  $\mu\text{M}$  ascorbic acid. The data represent the mean of three independent determinations. We removed the error bars of selected points in this figure (and in Figs. 2 and 5) to avoid confusing overlay of lines. In all cases, the important error bars which demonstrate differences are shown. Legend abbreviations: Ph = Photofrin®, Fe = ferrous iron, AscH<sup>-</sup> = ascorbate.

radical adduct (POBN/ $L_d$ ) spectrum. Instrument settings were: 10 G/21 s scan rate; 1.0 G modulation amplitude;  $1.0 \times 10^6$  receiver gain; 0.33 s time constant; and 40 mW nominal power [12].

### 3. Results and discussion

To probe for the production of free radicals in SCC-25 cells during Photofrin<sup>®</sup> photosensitization, we employed EPR spin trapping techniques using POBN. Photofrin<sup>®</sup> photosensitization of SCC-25 cells results in the production of membrane-derived lipid free radicals. The EPR spectrum observed consists of two radical species: the central doublet species ( $a^H = 1.8$  G) is the ascorbate free radical; the triplet of doublets is a POBN/ $L_d$  spin adduct ( $a^N = 15.65$  G,  $a^H = 2.71$  G), where  $L_d$  represents a lipid-derived free radical such as the ethyl or pentyl radical produced during lipid peroxidation (Fig. 1, inset) [9]. We reasoned that this free radical production could be increased if iron and ascorbate were present during illumination. Indeed, when iron and ascorbate were present during photosensitization, the production of lipid radicals increased significantly, an approximate four-fold increase at the 15 min time point ( $p < 0.01$ ) (Fig. 1). Control experiments with (1) pro-oxidant combination alone, (2) a complete system without light, (3) Pho-

tofrin<sup>®</sup> in the dark, or (4) Photofrin<sup>®</sup> with light without pro-oxidant combination, were all similar with a low lipid radical production.

Since iron and ascorbate increase lipid radical production during Photofrin<sup>®</sup> photosensitization, there might exist a concentration-dependence relationship with their application. Fig. 2(a) shows the effect of various concentrations of  $Fe^{2+}$  (in the presence of 100  $\mu$ M ascorbate) on the production of lipid radicals during PDT. An iron concentration of only 2  $\mu$ M produced a significant increase in lipid radical production during PDT as compared to 0  $\mu$ M added iron. However, concentrations of iron from 20  $\mu$ M to 200  $\mu$ M displayed no statistically significant concentration dependence. In the presence of a constant  $Fe^{2+}$  concentration (20  $\mu$ M), ascorbate displayed no significant concentration dependence from 0–50  $\mu$ M; however, 100  $\mu$ M ascorbate increased the lipid radical intensity over two-fold at the 15 min time point (Fig. 2(b)). We did not pursue concentrations of ascorbate above 100  $\mu$ M because of the possibility that an antioxidant activity will predominate at higher concentrations. These data suggest that the concentrations of iron and ascorbate, 20  $\mu$ M and 100  $\mu$ M respectively, are favorable *in vitro* concentrations of these compounds to enhance lipid radical formation.

If this increase in radical production is to be useful in enhancing the efficacy of Photofrin<sup>®</sup> photosensitization, then it should correlate with a decrease in cell survival. Indeed, the presence of iron and ascorbate during PDT significantly decreased cell survival at every time point after 5 min (at 10 min  $p = 0.008$ , at 15 min  $p = 0.039$ ) (Fig. 3). From the data in Figs. 1–3 we conclude that the presence of iron and ascorbate during PDT increases the *in vitro* production of

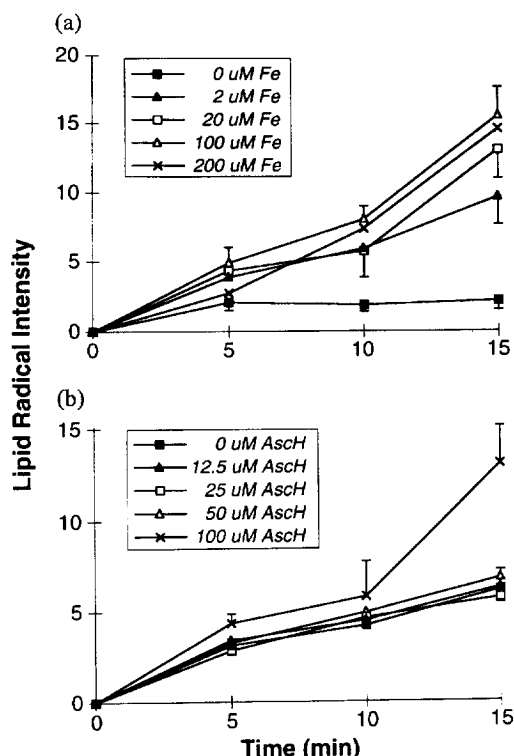


Fig. 2. Iron and ascorbate increase lipid radical intensity. (A) Fe studies: SCC-25 cells were manipulated as described in Fig. 1 in the presence of 0–200  $\mu$ M ferrous sulfate and 100  $\mu$ M ascorbic acid; or (b) ascorbate studies: in the presence of 0–100  $\mu$ M ascorbic acid in the presence of 20  $\mu$ M ferrous sulfate. The lipid radical intensity is in arbitrary units. The data represent the mean of three independent determinations.

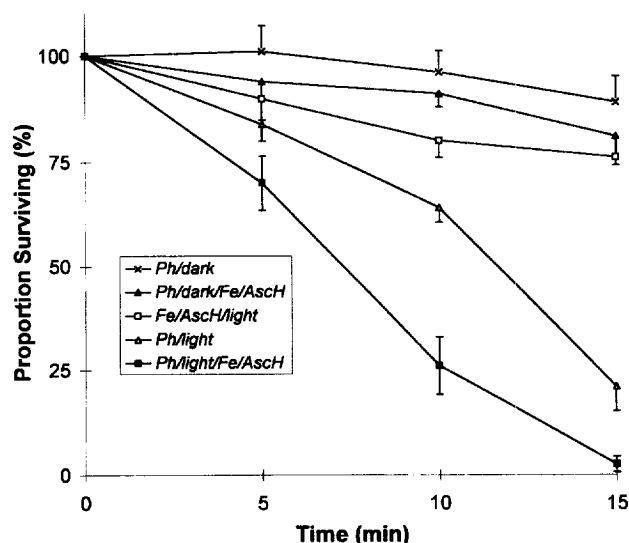


Fig. 3. Cell survival decreases when iron and ascorbate are present during Photofrin<sup>®</sup> photosensitization. SCC-25 cells were incubated as in Fig. 1. At the end of light exposure (or dark control), the cells were gently removed with trypsin/EDTA and assayed for cell survival using trypan blue dye exclusion. Each point and bar represent the mean and standard error of three independent determinations. Legend abbreviations: Ph = Photofrin<sup>®</sup>, Fe = 20  $\mu$ M ferrous sulfate, Asch<sup>-</sup> = 100  $\mu$ M ascorbate.

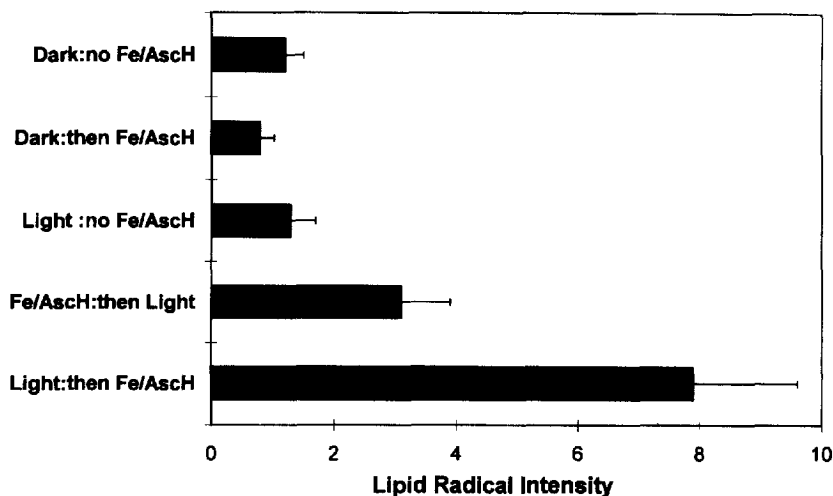


Fig. 4. The sequence of iron addition and light exposure influences total radical yield. SCC-25 cells were incubated with Photofrin<sup>®</sup> and manipulated as described in Fig. 1. Cells were illuminated for 5 min with visible light. Ferrous sulfate (20  $\mu\text{M}$ ) and ascorbate (100  $\mu\text{M}$ ) were added for a period of 1 min after: 5 min of illumination; during 5 min of illumination; or were not present during illumination. The lipid radical intensity is in arbitrary units. The data represent the mean and standard error of three independent determinations.

membrane-derived lipid radicals, and this increase correlates with enhanced cytotoxicity.

Photofrin<sup>®</sup> photosensitization produces singlet oxygen, which can react with unsaturated lipids to form lipid hydroperoxides. We postulated from our previously reported data on the L1210 cell line that PDT (without iron and ascorbate) would result in a build-up of membrane lipid hydroperoxides. The addition of iron and ascorbate to L1210 cells after photosensitization resulted in a burst of lipid radical production [10]. When iron and ascorbate are added to the SCC-25 cells after 5 min of PDT, there is also a burst of lipid radical production (two-fold over conditions where the pro-oxidant

combination is present from time zero) (Fig. 4). This increase in lipid radical production and its intensity correlates with the radical production achieved with approximately 11 min of light exposure (Fig. 1). These data add convincing evidence to support our basic hypothesis and suggest a possible technique for enhancement of the efficacy Photofrin<sup>®</sup> photosensitization in an *in vivo* model.

The concentration of Photofrin<sup>®</sup> used in most *in vivo* administered clinical trials has produced a skin sensitivity to natural light for up to one month after treatment [7]. Since iron and ascorbate in our model increases the efficacy of Photofrin<sup>®</sup> photosensitization, perhaps a lower dose of Photofrin<sup>®</sup> could be used to ameliorate side effects. The data in Fig. 5 represent *in vitro* photosensitization of SCC-25 cells with various concentrations of Photofrin<sup>®</sup> in the presence of 20  $\mu\text{M}$  iron and 100  $\mu\text{M}$  ascorbate. Photofrin<sup>®</sup> at 10  $\mu\text{g ml}^{-1}$  produced the same lipid radical intensity as 15  $\mu\text{g ml}^{-1}$ ; the radical intensity decreased at lower concentrations. Thus we obtained the same lipid radical EPR intensity with a one-third lower dose of Photofrin<sup>®</sup> when iron and ascorbate are present. Most importantly, there was no loss of cytotoxicity at the lower dose with iron and ascorbate (Fig. 6).

The significance of these observations, using a monolayer squamous cell carcinoma derived from a human head and neck tumor, lies in their potential applicability to *in vivo* photodynamic therapy of superficial cancers. From these data we conclude that the efficacy of *in vitro* PDT with Photofrin<sup>®</sup> can be increased by the topical application of the pro-oxidant combination of iron and ascorbate. Although therapeutic manipulation of tumor concentrations of  $\text{Fe}^{2+}$  and ascorbate prior to photosensitization may be a challenge for many cancers, it may be feasible for those cancers arising upon mucosal surfaces of the upper aerodigestive tract, especially the oral cavity. This study provides a rationale for the addition of topical iron and ascorbate to PDT, the selection of their con-

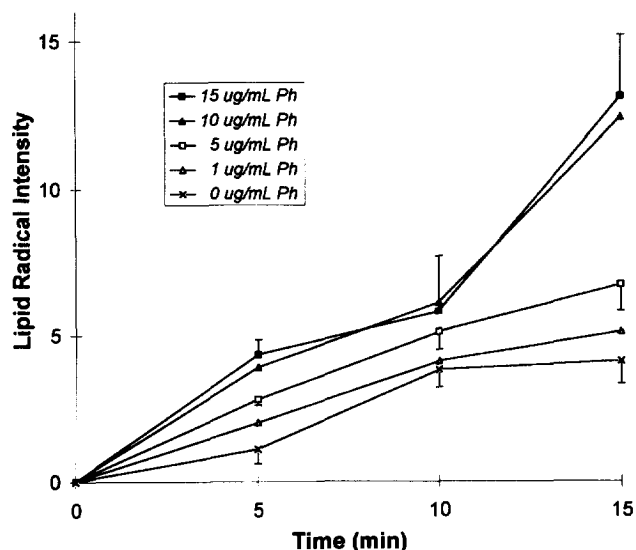


Fig. 5. Lipid radical increases with Photofrin<sup>®</sup> concentration in the presence of iron and ascorbate. SCC-25 cells were manipulated as described in the previous figures in the presence of 0, 1, 5, 10, and 15  $\mu\text{g ml}^{-1}$  of Photofrin<sup>®</sup> in the presence of 20  $\mu\text{M}$  ferrous sulfate and 100  $\mu\text{M}$  ascorbate. The lipid radical intensity is in arbitrary units. These data represent the mean and standard error of the mean of at least three independent determinations.

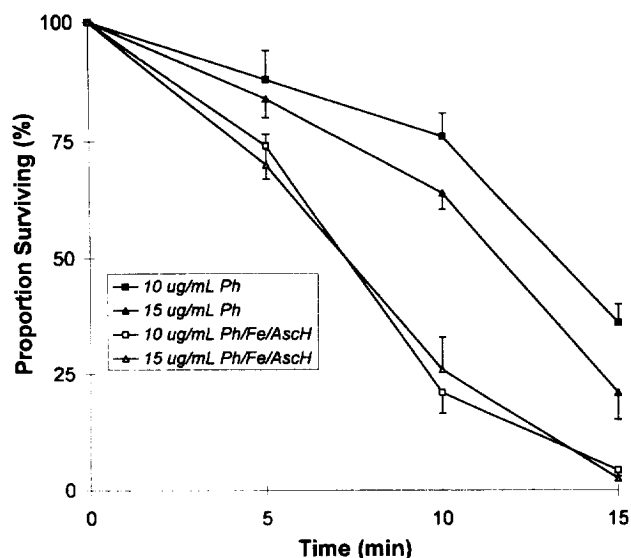


Fig. 6. The presence of iron and ascorbate during PDT reduces the concentration of Photofrin® needed. SCC-25 cells were incubated as in Fig. 1. At the end of light exposure the cells were gently removed with trypsin/EDTA and assayed for cell survival using trypan blue dye exclusion. Each point and bar represent the mean and standard error of three independent determinations.

centrations and the importance of the sequence of addition of the compounds in order to take advantage of the burst of oxidation resulting from the metabolism of pre-formed LOOH.

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