Effect of photodynamic therapy in combination with ionizing radiation on human squamous cell carcinoma cell lines of the head and neck

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Summary Photodynamic therapy (PDT) is a promising treatment modality for head and neck, and other tumours, using drugs activated by light. A second generation drug, 5-aminolaevulinic acid (5-ALA), is a precursor of the active photosensitizer protoporphyrin IX (PpIX) and has fewer side-effects and much more transient phototoxicity than previous photosensitizers. We have investigated the effect of 5-ALA mediated PDT in combination with γ-irradiation on the colony forming ability of several human head and neck tumour cell lines. The effect of treatments on the DNA cell cycle kinetics was also investigated. Our results indicate that the combination of 5-ALA mediated PDT and γ-irradiation results in a level of cytotoxicity which is additive and not synergistic. 5-ALA mediated PDT had no discernible effect on DNA cell cycle distributions. γ-irradiation-induced cell cycle arrest in G2 did not enhance the phototoxicity of 5-ALA. © 2000 Cancer Research Campaign

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Photodynamic therapy (PDT) is a method for the treatment of cancer that involves the administration of systemic or topical photosensitizing drugs that are preferentially taken up by the tumour and then activated in the presence of light to cause tissue destruction (Dougherty, 1988). Photodynamic therapy works by the generation of singlet oxygen that results in damage to cell membrane structures, microvascular ischaemia, and tissue necrosis. Numerous clinical trials of photodynamic therapy have been conducted over the past decade and PDT has been approved for clinical use in recurrent bladder carcinomas, obstructing oesophageal tumours, and early carcinomas of the bladder, oesophagus, stomach, and tracheobronchial tree. PDT has also been shown to provide curative treatment of early carcinomas of the head and neck, including the oral cavity, pharynx, and larynx (Biel, 1995; Feyh, 1996). Initially PDT was tested in advanced cancers of the head and neck that were untreatable or refractory to conventional therapy, however these trials produced only limited success (Schuller et al, 1984; Wile et al, 1984). A recent retrospective review of the clinical data available for the treatment of head and neck neoplasia using photodynamic therapy indicated that complete response rates of 89.5% are achievable for early squamous cell carcinoma of the head and neck (Biel, 1998). Based on a range of photosensitizers and treatment modalities, cure rates of 95% and 80% were obtained for carcinoma in situ and T1 squamous cell carcinoma of the vocal chord and oral cavity/tongue respectively (follow up 70 months) (Biel, 1998).

Most of the available photosensitizers, until recently, have been mixtures of porphyrins such as haematoporphyrin derivative and Photofrin (Quadralogic Technologies, Vancouver, Canada). The main problem with these first-generation photosensitizers is that of prolonged skin photosensitivity. Phototoxic incidences of 20–40% have been reported during follow-up of patients having received Photofrin, with a mean duration of skin photosensitivity exceeding 6 weeks (Dougherty et al, 1990).

The use of 5-aminolaevulinic acid (ALA) represents a different strategy in the administration of photosensitizers. ALA itself is not the photo-active drug, but rather it induces, in situ, the synthesis of a pure endogenous porphyrin called protoporphyrin IX (PpIX). The formation of PpIX forms part of the haem synthesis pathway and all nucleated cells that use oxidative metabolism are probably capable of forming this photosensitizer. However, malignant tissue appears to preferentially accumulate PpIX, forming the basis of photodynamic therapy in cancer (Battle, 1993; Kennedy and Pottier, 1994). Intravenous ALA is rapidly cleared from the body, with no PpIX fluorescence within the skin or other body organs detectable after 24 h (Kennedy et al, 1991).

Because of this reduced phototoxicity and excellent tumour localizing properties, ALA has been used with great success for the treatment of several neoplastic diseases, particularly of the skin, bladder and oral cavity. The results show very good clinical and cosmetic responses (Peng et al, 1997). However, the effects on thicker lesions (>1 mm) remain to be improved.

At present, the majority of head and neck tumours are treated conventionally with either surgery or ionizing radiation, although newer treatments are under investigation (van Dongen and Snow, 1997). Because many successful forms of cancer treatment rely upon a combination of treatment modalities, we have therefore investigated the treatment of head and neck carcinoma cell lines with various combinations of ionizing radiation and photodynamic therapy following administration of 5-aminolaevulinic acid.

MATERIALS AND METHODS

Cell lines and culture conditions

The three tumour cell lines used in this study; V134, V175, and SCC-61, are all human squamous cell carcinoma cell lines derived from tumours of the head and neck. The SCC-61 cell line, kindly donated by Dr AC Begg (Amsterdam, The Netherlands), has been previously described (Weichselbaum et al, 1984, 1988). The V134 (Champion et al, 1995) and V175 (Champion et al, 1997) cell lines were both derived at Velindre Hospital from patient tumour biopsy samples. Tumour cells were maintained as monolayer cultures in Dulbecco's Modified Eagles Medium (DMEM, Gibco, Paisley, UK) containing 10% fetal calf serum (Gibco, Paisley, UK), 100 U/ml penicillin (Sigma, Poole, UK 100 µg/ml streptomycin (Sigma, Poole, UK), and 0.4 µg/ml hydrocortisone (Sigma, Poole, UK).

Normal human fibroblasts were derived from skin biopsy material obtained during breast reduction surgery of non-cancer patients. Fibroblasts were maintained as monolayer cultures in DMEM containing 15% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin.

All cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were routinely subcultured by the addition of 0.02% trypsin/0.05% EDTA (Sigma, Poole, UK). Experiments were performed using cells harvested in log phase growth.

Irradiation

All irradiations were performed at room temperature in oxic conditions using a ^{137}Cs gamma source of 0.66 MeV energy. The dose rate was 1.2 Gy/min. Irradiations were usually completed within 15 min. Cells were irradiated as monolayers.

Clonogenic cell survival

Exponentially growing cells were irradiated in 25 cm^2 flasks in 5 ml culture medium. Triplicate flasks were set up for non-irradiated controls and for each radiation dose and each experiment was repeated three times. Surviving cells were allowed to grow for 10–14 days prior to fixing and staining. Resultant colonies were fixed in 4% formaldehyde in PBS and were stained with a 1% solution of crystal violet. Colonies consisting of more than 50 cells were counted.

Measurement of protoporphyrin IX accumulation

Confluent cell cultures were removed from their flasks by trypsinization and resuspended in serum supplemented medium to neutralize the trypsin. The cells were centrifuged at 150G for 5 min and the pellet resuspended in freshly prepared serum-free medium containing 1 mMol/l 5-aminolaevulinic acid (ALA, Sigma, Poole, UK) to give about 1×10^6 cells per 5 ml of medium. The cell suspensions were placed in 25 cm^2 flasks lined with a layer of 2% agar to prevent the cells attaching to the plastic. A paired flask of cell suspension in serum-free medium, but with no ALA was also prepared to act as a control. A 0.5 ml sample cell suspension was immediately removed from each of the paired flasks to measure the background fluorescence. Cell suspensions were incubated in the dark at 37°C in an atmosphere of 5% CO_2 in air. Samples were removed every hour from both sets of flasks to measure ALA-induced fluorescence. Flasks were gently shaken at these times but otherwise left undisturbed.

Cellular fluorescence was quantified with a FACScan flow cytometer (Becton-Dickinson, Oxford, UK). ALA-induced fluorescence was excited with an Argon laser emitting at 488 nm and emission was collected by a photomultiplier tube after passing through a 650 nm longpass filter. Data from 5×10^3 cells were recorded and processed using the LYSIS II software (Becton-Dickinson, Oxford, UK). Using side- and forward-scatter signals, debris was excluded from the final data. ALA-induced fluorescence was determined at various times by subtracting the fluorescence of the control cell suspensions from that of the ALA-incubated cells. The source of ALA-induced fluorescence was confirmed to be caused by the accumulation of protoporphyrin IX by checking the emission spectra of a sample of ALA-incubated cells with a standard protoporphyrin IX solution on a spectrophotometer (Perkin-Elmer, Beaconsfield, UK).

Photodynamic therapy

Exponentially growing cells were incubated for 4 hours in serum-free medium containing 1 mM/L ALA. An incubation period of 4 hours was chosen as in vivo studies of the fluorescence kinetics of ALA uptake in an animal model suggested this to be the optimum (Loh et al, 1993) and was further confirmed by our in vitro studies described in the present report.

Serum-free medium was used because protoporphyrin IX is lipophilic and rapidly diffuses out of the cell into medium-containing serum. The pH of the ALA dissolved in medium was maintained between 7.2 and 7.6. The cells were then incubated at 37°C in 5% CO_2 in the dark. After 4 h of incubation, each flask was then exposed to light from a tungsten-halogen lamp (Micromark, London, UK) for a specific time. The total spectral irradiance at the level of the cells, and in the presence of a water filter, was 50 mW/cm^2 (400–750 nm) measured using an Ophir Nova power meter (Ophir Optronics, Jerusalem, Israel) fitted with a black-body absorber pyroelectric head. These measurements indicated that the irradiance was constant over the area occupied by the tissue culture flask. Ultra violet light was minimal and measured at <1 mW/cm^2 with UVA <15 µW/cm^2. Infrared radiation was minimised using a 3.5 cm water filter between the cells and the light source. Flasks containing medium and exposed to the water-filtered light source for 20 min did not warm by >1°C. Experiments were performed on at least three separate occasions.

DNA contents

Cell cultures were removed from their flasks by trypsinization and resuspended in phosphate buffered saline (PBS). 125 µl of propidium iodide (0.4 mg/ml)/Triton X-100 (1% v/v) was added to 1 ml of cell suspension together with 50 µl Ribonuclease A (Sigma, Poole, UK 10 mg/ml). Cells were incubated at 37°C for 10 min prior to analysis with a FACScan flow cytometer (Becton-Dickinson, Oxford, UK). Propidium iodide fluorescence was excited with an Argon laser emitting at 488 nm and emission was collected by a photomultiplier tube after passing through a 650 nm longpass filter. Data from 5×10^3 cells were recorded and processed using the LYSIS II software (Becton-Dickinson, Oxford, UK). Using side- and forward-scatter signals, debris was excluded from the final data. Normal human lymphocytes were used to monitor instrument performance and to calibrate measurements of DNA ploidy. Where required, DNA histograms were deconvoluted according to the protocol of Watson et al (1987) and

Ormerod et al (1987), allowing accurate calculations of the number of cells in each of the cell cycle phases (G1, S, G2) to be made.

Clonogenic cell survival following combined PDT and γ-irradiation

Exponentially growing cells were irradiated in 25 cm² flasks in 5 ml culture medium either 24 hours before or 24 hours following a single, fixed 'dose' of PDT. The light dose used for the PDT was 3 minutes, which from our data resulted in a surviving fraction in the region of 0.6. Triplicate flasks were set up for non-irradiated controls and for each radiation dose and each experiment was repeated three times. Surviving cells were allowed to grow for 10–14 days prior to fixing and staining. Resultant colonies were fixed in 4% formaldehyde in PBS and were stained with a 1% solution of crystal violet. Colonies consisting of more than 50 cells were counted.

RESULTS

Time-dependent protoporphyrin IX accumulation

All cells incubated in serum-free medium containing 1 mM/l ALA over a 24 h time period showed a time dependent increase in fluorescence. However, flow cytometry of the various cells at 22–24 h showed a wide variation in their side- and forward-scatter signals. This suggests loss of viability and some degree of cell disintegration when cells are incubated in serum-free medium for this duration together with the forced prevention of cell attachment. In the case of the VB001 normal fibroblasts almost all of the cells were fragmenting due to apoptosis following the prolonged serum deprivation. Therefore, all further PDT experiments were performed under optimal conditions of 4 h incubation in 1 mM/l ALA. The fluorescence kinetics of the individual cell lines are summarised in Figure 1.

Clonogenic cell survival following photodynamic therapy

All of the cell lines examined had a dose-dependent clonogenic survival response to light, after 4 h of incubation in the presence of ALA (Figure 2). The 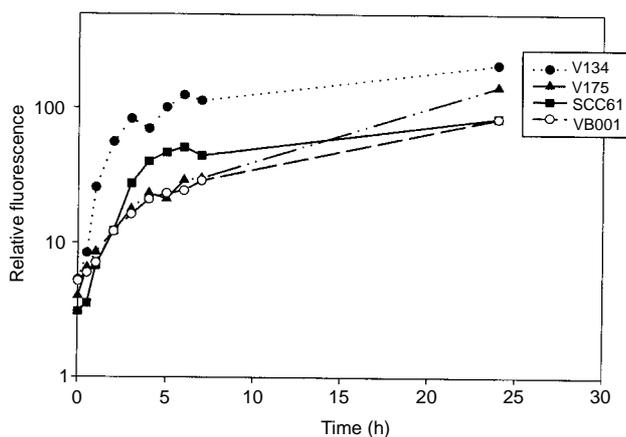data shown are from pooled experiments ($n \geq 3$). The data were fitted by a linear quadratic function for each cell line by means of a least squares Marquardt algorithm (Nash, 1979). In order to compare the responsiveness of the cell lines to ALA-mediated photodynamic therapy we used the surviving fraction following 3 min of PDT as an arbitrary value and termed this SF3. The SF3 value was derived by solving the equation $SF_3 = e(-(\alpha D + \beta D^2))$ for D = 3 min of light exposure. A fairly wide variation in PDT sensitivity was observed in these cell lines with SF_3 values of 0.54 (VB001), 0.67 (V134), 0.79 (V175) and 0.68 (SCC-61). The differing sensitivities were not correlated with the relative accumulation of protoporphyrin IX by the different cell lines during the 4 h incubation period.

Cells exposed to ALA but no light showed no increase in cell death. Similarly, there was no light-dose dependent response in the absence of ALA.

Clonogenic cell survival following γ-irradiation

The radiation dose–survival curves for the four cell lines are shown in Figure 3. The data shown are from pooled experiments ($n \geq 3$). The data were fitted by a linear quadratic function for each cell line by means of least squares Marquardt algorithm. Cell line characteristics and survival curve parameters are shown in Table 1. In order to compare the in vitro radiosensitivity of the cell lines we used the surviving fraction following treatment with 2 Gy of ionizing radiation (SF2) in accordance with previous studies (Brock et al, 1990; Girinsky et al, 1994; Champion et al, 1997). The SF-2 value was derived by solving the equation $SF2 = e(-(\alpha D + \beta D^2))$ for D = 2 Gy. A fairly wide variation in radiation sensitivity was observed in these cell lines, with SF2 values of 0.37 (SCC61), 0.61 (V175), 0.54 (V134), and 0.35 (VB001).

DNA cell cycle kinetics following photodynamic therapy or γ-irradiation

Histograms of cellular DNA content following various treatments are given in Figure 4. It can be seen that following 8 Gy of radiation the three tumour cell lines (V175, V134, SCC61) exhibit an accumulation of cells in the G2 phase of the cell cycle, whereas the normal fibroblasts (VB001) accumulate in both G1 and G2. This

Figure 1 Fluorescence kinetics of ALA-induced PpIX in the three head and neck cell lines and the normal fibroblasts used in this study. Data points represent the geometric mean of 5 × 10³ observations of cellular fluorescence
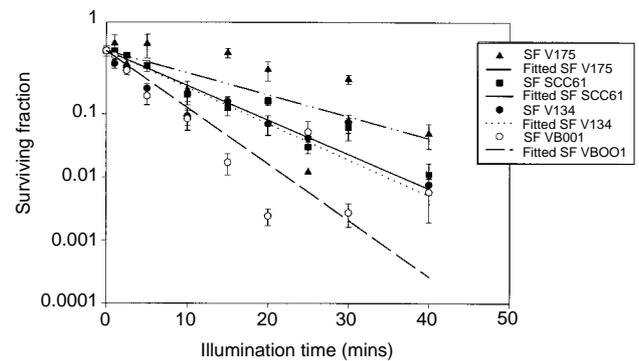

Figure 2 Clonogenic cell survival curves for the four cell lines used in this study, following exposure to 5-ALA-mediated photodynamic therapy. Data were fitted by a linear quadratic function. In this figure and all subsequent figures, the error bars represent one standard error of the mean and are shown if greater than the symbol
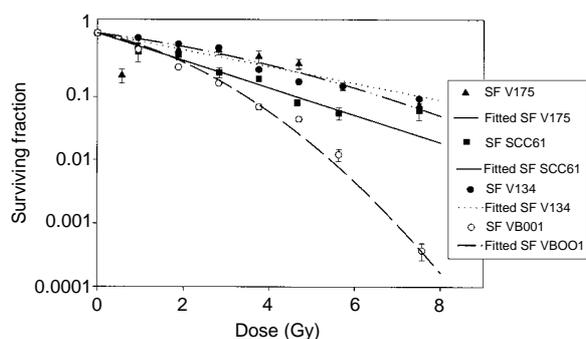
Figure 3 Clonogenic cell survival curves for the four cell lines following exposure to γ-irradiation
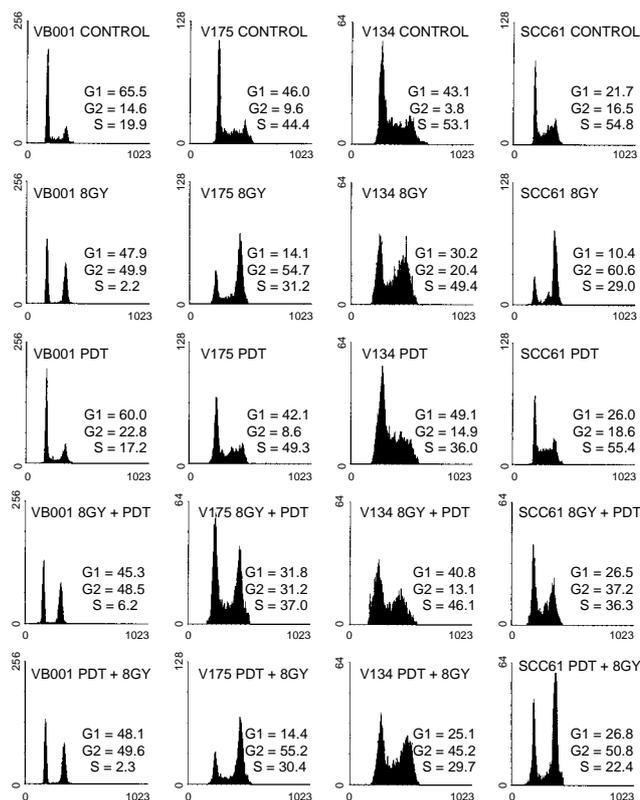

Figure 4 DNA histograms of the four cell lines used in this study. Cells were exposed to 8 Gy of ionizing radiation or 3 min of PDT or a combination of both treatments. Data represent distributions obtained from 5000 cells

observation is consistent with the suggestion that the three tumour cell lines have aberrant p53 function.

The cell line V134 shows a reduced accumulation in G2 following irradiation compared to the other cell lines and this correlates with increased radioresistance of this cell line. For the combination treatment where radiation is given prior to PDT the V134 showed a much reduced accumulation of cells in G2, indicating that cells are recovering from the cell cycle arrest during the 48 hour period between the initial radiation treatment and DNA measurement. PDT on its own had no discernible effect on cell cycle distributions of either tumour or normal cell populations.

Clonogenic cell survival following combined PDT and γ-irradiation

In order to investigate the cumulative effects of PDT and γ-irradiation on the cell lines, a fixed 'dose' of PDT was given either 24 hours before, or 24 hours following various doses of γ-irradiation. The light dose used for the PDT was 3 minutes, which from the above data resulted in a surviving fraction in the region of 0.6. The 24 hour delay between treatments was to allow any cell cycle arrest to be maximally in place before the cells underwent the second treatment.

The combined PDT/radiation-dose–survival curves for the four cell lines are shown in Figure 5. The data shown are from pooled experiments ($n \geq 3$). The data were fitted by a linear quadratic function as described above. Also shown on the figure are the theoretical survival curves for each cell line based on the assumption that the combination treatment is purely additive. This curve was obtained by multiplying the radiation dose–surviving fractions by the surviving fraction obtained following 3 min of PDT. It can be seen that none of the cell lines exhibit survival curve parameters which are significantly different from these theoretical curves. The survival curve parameters are summarised in Table 2.

DISCUSSION

This study has demonstrated the effect of photodynamic therapy used in combination with ionising radiation on clonogenic cell survival of head and neck tumour cell lines. Our results indicate that under the conditions employed in this study the cumulative effects of combined treatments are purely additive.

It has previously been shown that tumour cell sensitivity to PDT varies during the cell cycle (Wyld et al, 1998) with cells in S-phase and G2 being more sensitive. In the case of 5-ALA this is associated with an increased accumulation of PpIX during S-phase and G2. There is also an inverse relationship between PpIX synthesis and cellular iron availability (Rittenhouse-Diakun et al, 1995). S-phase cells may be relatively iron-depleted because of intracellular sequestration of iron by ribonucleotide reductase during DNA synthesis (Brown et al, 1969; Reichard and Ehrenberg 1983) and this may play a role in the increased PpIX synthesis during

Table 1 Summary of cell characteristics and clonogenic survival data for cell lines treated with ionizing radiation or photodynamic therapy

| Cell line | Doubling time (h)[a] | Clonogenic plating efficiency (% + 1 SE)[a] | Ploidy[a] | γ-radiation | | | PDT SF3 |
				SF^2	$\alpha(Gy^{-1})$ (+1SE)	$\beta(Gy^{-2})$ (+ 1 SE)	
VB001	nd	15.1 ± 1.2	normal diploid	0.35	0.344 ± 0.056	0.093 ± 0.009	0.54
V134	28.8	5.67 ± 0.25	polyploid	0.55	0.304 ± 0.067	0.000 ± 0.011	0.67
V175	35.7	2.82 ± 0.23	hyperdiploid	0.61	0.202 ± 0.051	0.022 ± 0.008	0.79
SCC-61	32.75	24.13 ± 1.18	hyperdiploid	0.37	0.619 ± 0.065	0.000 ± 0.011	0.68

[a]Data from Champion *et al* (1997).

Table 2 Summary of clonogenic survival data for cell lines treated with ionizing radiation followed by photodynamic therapy and vice-versa

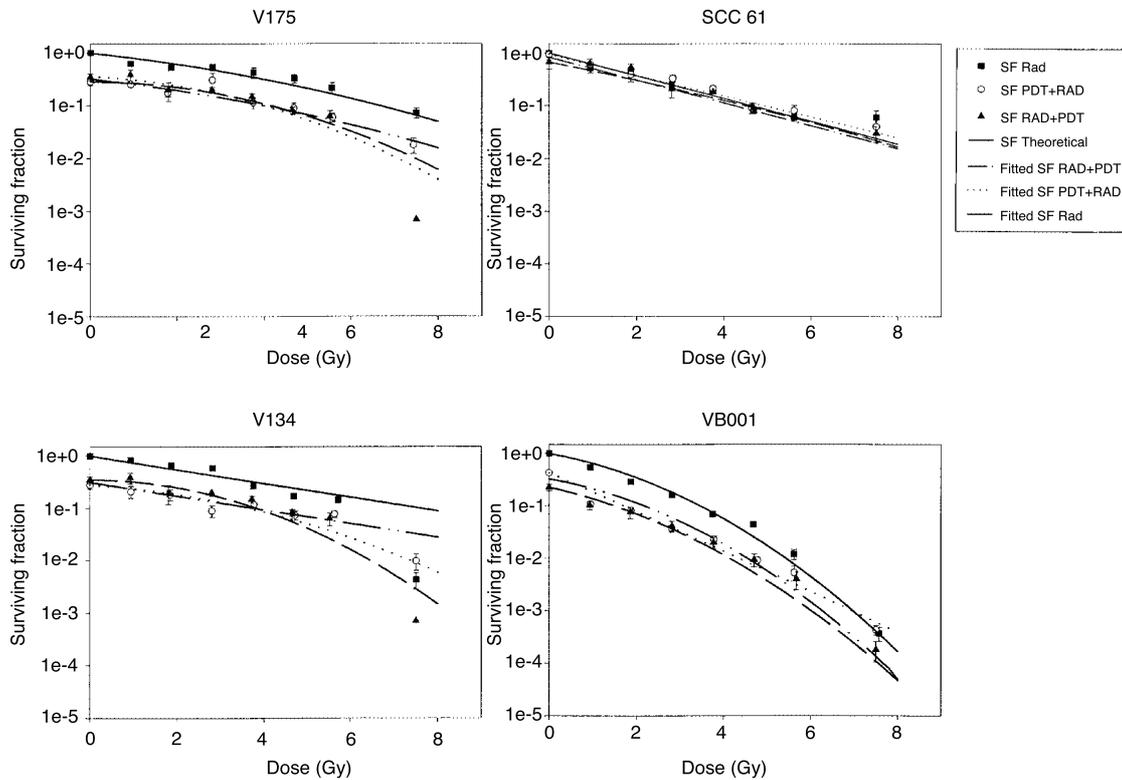| Cell Line	γ-Radiation + PDT			PDT + γ-Radiation			Theoretical SF$_2$
	SF$_2$	α(Gy^{-1}) (+1 SE)	β(Gy^{-2}) (± 1 SE)	SF$_2$	α(Gy^{-1} (± 1 SE)	β(Gy^{-2}) (± 1 SE)	
VB001	0.07	0.00	–	0.078	0.676 ± 0.063	0.023 ± 0.012	0.114
V134	0.25	0.00	–	0.184	0.114 ± 0.091	0.046 ± 0.015	0.170
V175	0.26	0.144 ± 0.082	0.035 ± 0.014	0.327	0.00	0.060 ± 0.013	0.266
SCC-61	0.35	0.376 ± 0.070	0.011 ± 0.012	0.496	0.314 ± 0.061	0.011 ±0.010	0.351

Figure 5 Clonogenic cell survival curves for the four cell lines. Cells were treated with ionizing radiation, PDT followed 24 h later by ionizing radiation, or radiation followed 24 h later by PDT. Also shown on the figure are the calculated (theoretical) survival curves assuming any combined effect to be additive in nature

S-phase. It has further been observed that rapidly proliferating cells with increased S-phase fractions produce more PpIX and are more PDT-sensitive than slowly proliferating cells (Schick et al, 1995). This relationship between PDT sensitivity and growth rate may also be due to intracellular iron regulation (Pourzand et al, 1999). These marked fluctuations in PDT sensitivity with growth rate may account for the wide scatter observed in our measurements of clonogenic cell survival following PDT.

Mitomycin C has been demonstrated to potentiate photodynamic therapy both at the cellular level (Ma et al, 1992a, 1993a; Datta et al, 1997) and in vivo (Baas et al, 1994, 1996). This potentiation of PDT is associated with a mitomycin-C induced cell cycle arrest in late S-phase/G2 (Ma et al, 1992b). It would therefore be reasonable to expect that a radiation induced cell cycle arrest in G2 would also enhance the effectiveness of PDT. However, we have found no evidence to support this hypothesis and the sensitivity of our cell lines to PDT did not correlate with their relative accumulation of PpIX. Following radiation treatment, the three tumour cell lines used in this study (V175, V134, SCC61) exhibited a cell

cycle arrest in G2, whereas the normal fibroblasts arrested in both G1 and G2. This observation is consistent with the suggestion that the three tumour cell lines have aberrant p53 function (Kastan et al, 1991, Kuerbitz et al, 1992), and it is encouraging that this does not appear to be conferring any inherent resistance to the photodynamic treatment.

There have been a number of previous attempts to examine the interaction of PDT and ionizing radiation. However the results presented are conflicting, with some authors presenting evidence of a synergistic interaction (Boegheim et al, 1987; Kostron et al, 1986; Berg et al, 1995), while other investigations show simple additive interaction (Bellnier and Dougherty 1986; Ben-Hur et al, 1988; Schnitzhofer and Krammer 1996). The picture is further complicated by some studies providing evidence of a synergistic effect or an antagonistic effect depending upon the conditions employed within a given system (Ma et al, 1993b, Berg et al, 1995). The causes for the conflicting literature on the combination of radiotherapy and PDT are unclear. Prinsze et al (1992) suggested that cell line differences in the sensitivity to PDT

induced inhibition of DNA repair can explain the conflicting results. Other authors (Kavarnos et al, 1994; Ma et al, 1993b; Berg et al, 1995) suggest that the variations observed in the interaction of PDT and ionizing radiation depend on dose and variation in timing between the two treatments.

In vivo, photodynamic therapy is usually accompanied by severe microvascular changes (Nelson et al, 1987) associated with endothelial damage, microcirculatory stasis, platelet aggregation and haemorrhage, resulting in a coagulation necrosis (Bugelski et al, 1981; Selman et al, 1984; Star et al, 1986; Chaudhuri et al, 1987). These additional effects make it difficult to extrapolate in vitro studies on cell lines to the clinical situation where the combined approach of radiotherapy and photodynamic therapy may have quite different effectiveness than that presented in this study.

In conclusion, head and neck tumour cells were shown to accumulate in G2/M phase of the cell cycle after γ-irradiation. When γ-irradiation was combined with PDT the two treatments acted in an additive manner regardless of the order in which they were given. The interaction between γ-irradiation and PDT may be complicated by dose and timing variations between treatments, and further complicated by fractionated dosing of both the γ-irradiation and PDT. These variable interactions are worthy of further investigation, with a view to defining a role for combined radiotherapy and PDT in tumour therapy.

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