

Cell cycle phase influences tumour cell sensitivity to aminolaevulinic acid-induced photodynamic therapy in vitro

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Summary Photodynamic therapy (PDT) is a form of cancer treatment based on the destruction of cells by the interaction of light, oxygen and a photosensitizer. Aminolaevulinic acid (ALA) is the prodrug of the photosensitizer protoporphyrin IX (PpIX). ALA-induced PDT depends on the rate of cellular synthesis of PpIX, which may vary with cell cycle phase. This study has investigated the relationship between cell cycle phase, PpIX generation and phototoxicity in synchronized and unsynchronized bladder cancer cells (HT1197). In unsynchronized cells, relative PpIX fluorescence values (arbitrary units) were significantly different between cell cycle phases after a 1-h ALA incubation (G_1 , 24.8 ± 0.7 ; S-phase, 32.7 ± 0.8 , $P < 0.05$; G_2 , 35.4 ± 0.8 , $P < 0.05$). In synchronized cells after a 1-h ALA incubation, cells in G_1 produced less PpIX than those in S-phase or G_2 [6.65 ± 1.1 ng per 10^5 cells compared with 15.5 ± 2.1 ($P < 0.05$), and 8.1 ± 1.8 ng per 10^5 cells (not significant) respectively] and were significantly less sensitive to ALA-induced PDT (% survival, G_1 , 76.2 ± 8.3 ; S-phase 49.7 ± 4.6 , $P < 0.05$; G_2 , 44.2 ± 2.4 , $P < 0.05$). This differential response in tumour cells may have implications for clinical PDT, resulting in treatment resistance and possible failure in complete tumour response.

Keywords: cell cycle; aminolaevulinic acid; photodynamic therapy; in vitro

Photodynamic therapy (PDT) is an anti-cancer therapy that damages cells by the generation of reactive oxygen species due to the interaction of light, oxygen and a photoactive chemical (Weishaupt et al. 1976). It has been shown to be effective in the treatment of a wide variety of neoplasms including bladder cancer (Kriegmair et al. 1996) and carcinoma in situ of the bladder (D' Hallewin and Baert, 1995), oesophageal cancer (Sibille et al. 1995), a variety of skin cancers (Dougherty et al. 1978) and gastric cancer (Hayata et al. 1985). However, there is often a substantial recurrence rate or incomplete response rate possibly because of focal areas of under treatment (Hayata et al. 1985). Histological examination of treated tissues has revealed small islands of viable cells that may serve as foci for these recurrences (Suzuki et al. 1987). This is thought to be due to inadequate light penetration to all areas of the cancer, or to resistant cells in areas of hypoxia (Moan and Sommer, 1985). Intrinsic cellular resistance to PDT (for example due to effective free radical scavenging) or inadequate photosensitizer levels within the cell may also be important factors.

Aminolaevulinic acid (ALA) is the prodrug of the photosensitizer protoporphyrin IX (PpIX). After ALA administration, cells generate PpIX via the haem biosynthetic enzyme system (Berlin et al. 1956). Sensitivity to ALA-induced PDT depends, in part, on the PpIX generation rate within the cell. The metabolic activity of a cell may vary with the phase of the cell cycle (Kaczmarek, 1986), and the activity of cellular enzymes may fluctuate in a cell

cycle-dependent manner (Churchill and Studzinski, 1970). Therefore, cells in certain phases of the cell cycle may produce different amounts of PpIX resulting in differential levels of PDT sensitivity. Actively proliferating cells produce more PpIX on incubation with ALA than quiescent cells (Schick et al. 1995). This may be related to the intracellular availability of iron, with iron-depleted cells generating PpIX more rapidly (Rittenhouse-Diakun et al. 1995). S-phase progression can be inhibited by iron withdrawal (Nocka and Pelus, 1988), possibly because of the iron requirements of DNA synthetic enzymes such as ribonucleoside diphosphate reductase, which requires two iron atoms for activity (Brown et al. 1969). Thus, it might be expected that the iron requirements of S-phase cells would deplete intracellular stores, allowing increased production of PpIX. It is known that transferrin receptor expression on the cell surface is maximal in the G_2 , S- and M-phases of the cell cycle (indicating relative iron depletion) and falls by a factor of 4 during G_1 (Necker and Cossman, 1983). Fukuda et al (1993) studied the relationship between the phase of the cell cycle and the rates of PpIX generation using an epithelial cell line and serum withdrawal as a means of inducing cell synchronization. This study did not demonstrate any significant differences between cell cycle phases, although a trend towards higher PpIX production by G_2 cells was noted.

The variability in PDT sensitivity with the cell cycle may be analogous to that seen using radiotherapy. Radiotherapy, like PDT, induces cytotoxicity through the generation of reactive oxygen species (Hewitt and Wilson, 1959). Both radiotherapy and PDT are known to cause DNA damage (Gomer, 1980), although PDT causes fewer DNA strand breaks per effective dose unit than radiotherapy (Moan et al. 1983). Cells are most sensitive to radiotherapy during mitosis, late G_1 and early S-phase, and are relatively resistant in late S-phase and early G_1 (Terasima and

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Tolmach, 1963). Cells in G_2 are also relatively radiosensitive (Humphrey and Dewey, 1965). Similar effects have been observed with haematoporphyrin derivative (HPD)-induced PDT, when cells are least sensitive in early G_1 and are most sensitive in S-phase (Christensen et al, 1981).

The aims of this project were, therefore, to determine whether there is any variation in the rates of PpIX generation between different cell cycle phases and to investigate whether this is related to differential PDT sensitivity.

MATERIALS AND METHODS

Cell culture

Human bladder cancer cells (HT1197, Rasheed et al, 1977) were obtained from the European Collection of Animal Cell Cultures (Porton Down, UK, ECACC). Cells were cultured at 37°C in air supplemented with 5% carbon dioxide in Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 10% newborn calf serum (Gibco) and 1% penicillin and streptomycin solution (10 000 IU ml⁻¹ and 10 000 µg ml⁻¹, respectively, Gibco).

Cell cycle duration: bromodeoxyuridine (BrdU) pulse labelling

A modified BrdU pulse-labelling technique was used to determine cell cycle duration (Dolbeare and Seldon, 1994). BrdU is a thymidine analogue, taken up exclusively by cells in S-phase, which can then be detected by specific fluorescent-labelled monoclonal antibodies. Exponentially growing cells (2×10^6) were incubated with 10 µM bromodeoxyuridine (Sigma Chemicals, UK) in complete media for 30 min. The cells were then washed twice in phosphate-buffered saline (PBS, Sigma Chemicals, UK) and incubated in complete media without BrdU. At 3-h intervals for 15 h cells were removed from flasks by trypsinization (trypsin 0.05% and EDTA 0.02%, Gibco) and fixed in 1% paraformaldehyde (Sigma Chemicals, UK) for 5 min at room temperature. The cells were then permeabilized in 70% methanol at 4°C for 24 h. DNA was denatured with 2 M hydrochloric acid with 1 mg ml⁻¹ pepsin A (derived from porcine stomach mucosa, Sigma Chemicals, UK) for 1 h at room temperature. The acid was neutralized with 0.1 M borax solution (Sigma Chemicals, UK). Cells were incubated with anti-BrdU antibody (25 µg ml⁻¹ monoclonal mouse anti-BrdU, Dako, UK) for 30 min at 37°C followed by a second incubation with a fluorescein isothiocyanate (FITC) conjugated second antibody (7 µg ml⁻¹ goat anti-mouse, Caltag Laboratories, CA, USA) for a further 30 min at 37°C. Cells were washed with PBS with 1% fetal calf serum (Gibco) between antibody additions to reduce non-specific antibody binding. Cellular DNA was then labelled with propidium iodide (PI, 50 µg ml⁻¹, Sigma Chemicals, UK) to which RNAase (Sigma Chemicals, UK) was added to denature any double-stranded RNA before being run on the cytometer (Beckton Dickinson FACSsort). Cells were analysed with excitation at 488 nm and emission at 630 (± 22) nm for propidium iodide and 530 (± 30) nm for FITC. Three repeats were performed.

Cell synchronization

This was based on the method of Stein et al (1995). Exponentially growing cells were treated with complete media containing 2 mM thymidine (Sigma Chemicals, UK), which inhibits cell cycle

progression for cells in S-phase and blocks entry of further cells into S-phase. The treatment time corresponded to the total duration of G_2 , M-phase and G_1 , as determined by BrdU pulse labelling, to allow cells in G_2 , M-phase and G_1 to accumulate at the G_1 /S boundary. The thymidine block was then removed, the cells washed and replaced in media containing 24 µM deoxycytidine, which replenishes the nucleotide precursor pools, and incubated for a time corresponding to the duration of S-phase. Cells previously blocked in S-phase and those held at the G_1 /S boundary then pass through S-phase and into G_2 . The thymidine block is then reapplied for the $G_2 + M + G_1$ time interval, to allow all cells to progress to the G_1 /S boundary but prevent entry into S-phase. On release of this second block with deoxycytidine, all of the cells should progress through the cell cycle in a synchronized manner. To confirm that synchronization had occurred, synchronized cells were labelled with BrdU, sampled at 3-h intervals and processed using flow cytometry. This allowed quantification of the proportion of cells in each phase of the cell cycle at different times after release of the block. The effect of inclusion of 1 mM ALA (Sigma) in the culture media of synchronized cells during cell cycle progression was also studied.

The resultant synchronized DNA histograms were analysed using a curve-fitting program (ModFit LT, Verity Software House), which determined the percentage of cells in each phase of the cell cycle at each time point. (Five repeats of each experiment were performed.)

PpIX determination

This method has been described in a previous publication (Wyld et al, 1997). In brief, flasks of synchronized cells were obtained as above. At times corresponding to S-phase, G_2 /M and G_1 , cells were incubated with 1 mM ALA in complete medium for 1 or 4 h, in the dark, at 37°C. PpIX concentrations in the cells and the media were then determined by spectrofluorimetry (excitation at 406 nm, emission at 604 nm, Perkin-Elmer LS-3 fluorescence spectrometer). Values were expressed as either ng PpIX per 10⁵ cell or ng PpIX per µg cellular protein. The cell count was determined using haemocytometry (Freshney, 1987) and the protein content by the micro-Lowry method (Lowry et al, 1951). Six triplicate repeats were performed.

MTT assay

This technique allows quantification of cell survival after a cytotoxic insult. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma Chemicals, UK) is metabolized by the mitochondrial enzyme, lactate dehydrogenase to a violet-coloured formazan product in direct proportion to the number of cells present over a predetermined range (Stratford and Stephens, 1989). The optical density of the product is quantified spectrophotometrically on a 96-well-plate reader. MTT, 0.5 mg ml⁻¹, was added to each well of a 96-well plate and incubated for 2 h. The medium was then removed and replaced with 200 µl of dimethyl sulphoxide (DMSO) (Sigma, UK) to solubilize the formazan. The plate was then read on an ELISA plate reader (Anthos Labtec Instruments) at 550 nm absorbance. The initial plating density was determined by plating serial cell dilutions into the wells of a 96-well plate and comparing the direct haemocytometer count with the colour density produced. All further experiments used a cell density within the linear portion of the curve.

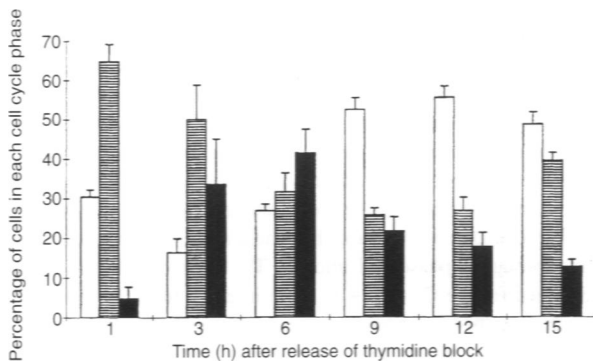


Figure 1 Percentage of cells in different cell cycle phases after release of thymidine block. Data shown are the means + s.e.m. $n = 5$. □, G₁; ▨, S; ■, G₂

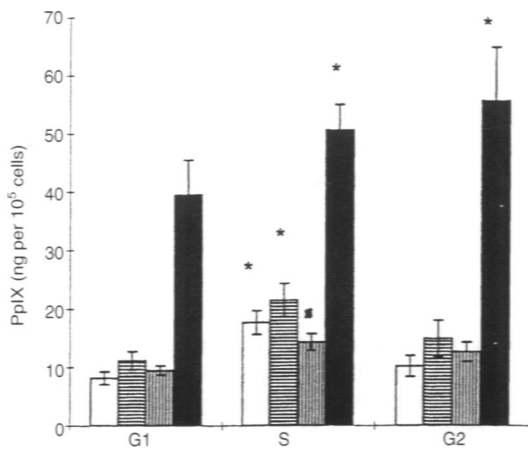


Figure 2 PpIX generation at different phases of the cell cycle in ng per 10^5 cells. Data represent the means + s.e. *Significantly different from G₁ value, $P < 0.05$. □, intracellular PpIX, 1-h ALA; ▨, total PpIX, 1-h ALA; ■, intracellular PpIX, 4-h ALA; ■, total PpIX, 4-h ALA

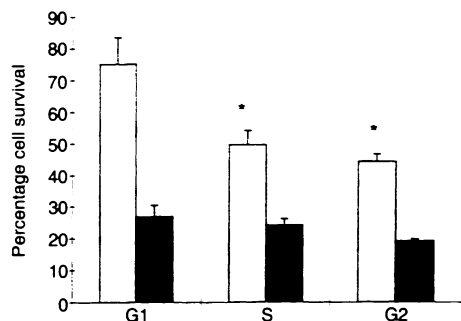


Figure 3 Percentage of cell survival after PDT (after a 1-h ALA incubation) in different cell cycle phases. □, light dose 0.5 J cm^{-2} ; ■, light dose 1.5 J cm^{-2}

PDT sensitivity

This method has been described in a previous publication (Wyld et al. 1997). Cells were plated into 96-well plates at a cell density of $2 \times 10^4 \text{ ml}^{-1}$ and synchronized as above. At times corresponding to S-phase, G₂ and G₁ of the cell cycle, 1 mM ALA was added to the cells and incubated for 1 or 4 h in the dark at 37°C. The cells were then exposed to violet light (350–450 nm), at a total dose of

0.5 J cm^{-2} or 1.5 J cm^{-2} . The medium was then replaced with standard complete medium, the cells were returned to the incubator for 24 h and then an MTT assay was performed. Controls for light alone, ALA alone and neither light nor ALA were also performed. Percentage survival was determined according to the following equation:

$$\text{Survival (\%)} = \frac{\text{Optical density of treated well}}{\text{Optical density of control well}} \times 100$$

Six triplicate repeats were performed.

Dual-labelling of unfixed, unsynchronized cells

Unsynchronized, exponentially growing cells were incubated with 1 mM ALA for either 1 or 4 h plus $10\text{--}25 \mu\text{g ml}^{-1}$ bisbenzamide (Hoechst 33342, Sigma Chemicals, UK) in complete media for 1 h. The cells take up bisbenzamide in proportion to their DNA content (Crissman, 1995). The cells were then trypsinized and resuspended in media at a density of $1 \times 10^6 \text{ cells ml}^{-1}$ and analysed on the flow cytometer (Beckton Dickinson, FACS Vantage). PpIX excitation was at 488 nm and emission at 600 nm and bisbenzamide excitation at 300 nm (ultraviolet) and emission at 500–550 nm. This produced a bivariate histogram of PpIX fluorescence (on the y-axis) against DNA (HO33342) fluorescence (on the x-axis). The mean PpIX fluorescence at DNA contents corresponding to G₁, S-phase and G₂ were then calculated. Six repeats of this experiment were performed for both 1- and 4-h ALA incubations.

Statistical analyses

All data are represented as means plus or minus the standard error of the mean (SEM). Statistical analyses were carried out using an initial analysis of variance (Kruskal–Wallis), followed by a Mann–Whitney *U*-test. Statistical significance was accepted if $P < 0.05$.

RESULTS

Cell characteristics

HT1197 cells were used between passages 19 and 40. The BrdU-labelling index of unsynchronized cells was $36.8 \pm 1.3\%$ (% of S-phase cells). The cell cycle distribution for unsynchronized cells, according to their relative DNA (propidium iodide) content, after ModFit analysis, was S-phase $36.4 \pm 2.4\%$, G₁ $43.8 \pm 2.2\%$ and G₂ $19.7 \pm 2.9\%$. The coefficient of variation for the G₁ peak of the DNA histograms was 7.06 ± 0.76 ($n = 6$), which implies adequate quality DNA staining.

Cell cycle progression

Using BrdU pulse labelling the duration of the different phases of the cell cycle were calculated to be: S-phase 6 h; G₂ 3 h and G₁ 6 h with an overall cell cycle duration of 15 h. This is in agreement with the population doubling time ($15.1 \pm 2.9 \text{ h}$) previously determined in this laboratory.

Cell synchronization

The mean percentage of cells in each phase of the cell cycle at each time point as calculated by ModFit analysis of the DNA

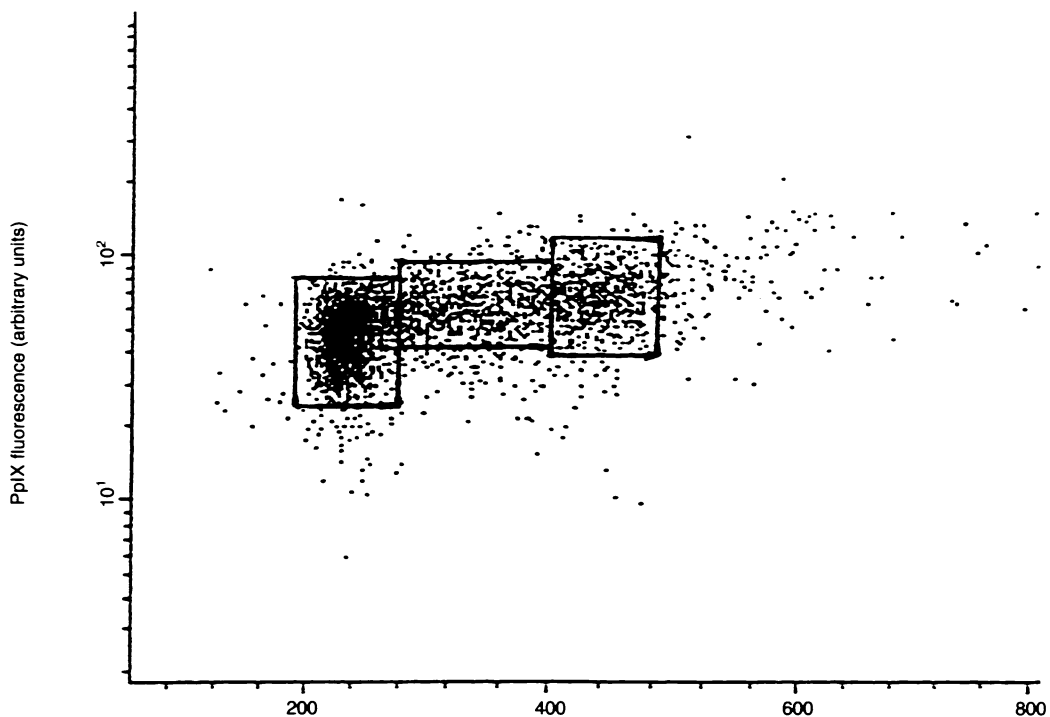


Figure 4 Bivariate histogram of PpIX fluorescence (y-axis) against DNA content (bisbenzamide fluorescence) after a 4-h incubation with ALA

histograms are shown in Figure 1. From these data, the optimal sampling times for each phase of the cell cycle after release of the thymidine block were determined. The percentage of BrdU-labelled cells can also be used as an estimate of the efficiency of synchronization in the S-phase period of the cell cycle, i.e. the 1- and 3-h samples. The percentage of BrdU-labelled cells at 1 and 3 h were 71 ± 3.3 and 77 ± 2.8 , respectively, suggesting a good level of cell synchronization. The total cell cycle duration was 15 h. The 4-h ALA treatment times were as follows: S-phase from 1 to 5 h, G_2 from 5 to 9 h and G_1 from 10 to 14 h. For 1-h ALA treatment, the S-phase was from 1 to 2 h, G_2 from 5 to 6 h and G_1 from 11 to 12 h. Incubation of cells in media containing 1 mM ALA for the 15-h duration of this experiment had no detectable effect on cell cycle progression (data not shown).

Protoporphyrin IX generation

After incubation of synchronized cells with ALA for 1 h, S-phase cells produced significantly more intracellular PpIX per 10^5 cells than G_2 or G_1 cells (15.5 vs 8.1 , $P < 0.05$ and 6.6 , $P < 0.05$ respectively) and significantly more total (cellular plus media PpIX) PpIX 10^5 cells than G_2 and G_1 cells (17.8 vs 11 , $P < 0.05$ and 9.6 , $P < 0.05$ respectively, Figure 2). When PpIX generation was calculated per μg of cellular protein, a similar pattern was seen with intracellular PpIX being significantly greater in S-phase cells than in G_2 or G_1 cells (0.12 vs 0.98 , $P < 0.05$, and 0.1 , $P < 0.05$ respectively).

After a 4-h incubation of synchronized cells with ALA, total PpIX per 10^5 cells was significantly less in G_1 than in G_2 and S-phase cells (39.5 vs 56 , $P < 0.05$ and 51 , $P < 0.05$ respectively), and intracellular PpIX was significantly less in G_1 than in S-phase cells (9.5 vs 12.7 , $P < 0.05$ respectively, Figure 2). When PpIX was calculated per μg of protein after a 4-h incubation with ALA,

no significant differences were noted between cell cycle phases (data not shown).

PDT sensitivity

After a 1-h incubation with ALA and light administration, cell survival was greatest in G_1 cells and least with cells in G_2 at both light doses studied (Figure 3). With the 0.5 J cm^{-2} light dose, G_1 survival was significantly greater than G_2 and S-phase (76.2 vs 44.2 , $P < 0.05$ and 49.7 , $P < 0.05$ respectively). At the higher light dose no significant differences were observed. After a 4-h incubation with ALA followed by PDT, no significant difference was noted between cell cycle phases (data not shown). Controls for light alone and ALA alone showed no significant toxicity compared with the no light, no ALA control.

Dual labelling

After a 1-h incubation with ALA and bisbenzimidazole, DNA histograms were obtained (mean coefficient of variation of the G_1 peak of 10.3 ± 0.9) and PpIX fluorescence was detected. The bivariate histograms showed the G_1 cells to have a wider range of PpIX fluorescence than the other two phases of the cell cycle with a significantly lower mean (G_1 , 24.8 ; S, 32.7 ; $P < 0.05$ and G_2 , 35.4 , $P < 0.05$). After a 4-h incubation with ALA and 1 h with bisbenzimidazole, these cell cycle phase differences were similarly significantly different (G_1 , 46.5 ; S-phase, 55.9 , $P < 0.05$; and G_2 , 65.1 , $P < 0.05$). The PpIX levels were significantly ($P < 0.05$) greater after a 4 h ALA incubation than after 1 h (55.2 ± 4.7 and 28.1 ± 0.7 respectively). An example of a PpIX/bisbenzimidazole histogram is shown in Figure 4 and mean cell cycle-specific PpIX fluorescence values are shown in Figure 5.

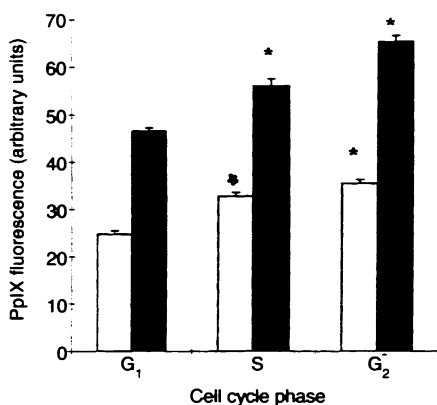


Figure 5 PpIX fluorescence in different cell cycle phases using unsynchronized cells labelled with bisbenzamide and PpIX. Data shown are the mean + s.e.m. *Significantly different from G₁ value, $P < 0.05$. □, 1-h ALA incubation. ■, 4-h ALA incubation

DISCUSSION

This study has demonstrated increased levels of PpIX in cells in G₂ and S-phase of the cell cycle and a corresponding increase in sensitivity to PDT under certain conditions.

Cells in G₂ and S-phase of the cell cycle produce greater levels of PpIX than cells in G₁ after exposure to ALA in both synchronized and unsynchronized cells. This is in agreement with the observation that S-phase cells may be relatively iron depleted because of intracellular competition for iron from enzymes involved in DNA synthesis (Brown et al, 1969) and the known inverse relationship between PpIX synthesis and cellular iron availability (Rittenhouse-Diakun et al, 1995). It may also, in part, explain the observation that rapidly proliferating cells with an increased S-phase fraction produce more PpIX and are more PDT-sensitive than slowly proliferating cells (Schick et al, 1995). Differential PpIX generation was most apparent when PpIX levels were calculated per 10⁶ cells rather than per µg of cellular protein. This may reflect variations in the cellular content of protein at different phases of the cell cycle, as total cellular protein tends to increase linearly through the cell cycle from G₁ to G₂ (Stein and Borun, 1972).

Fukuda et al (1993) studied ALA-induced PpIX generation in an epithelial cell line after synchronization by serum withdrawal and readdition. No significant differences in PpIX production between cell cycle phases were demonstrated, although it is not known what percentage of the cells were synchronized by the serum withdrawal process. A low level of synchronization may have diluted any differences between cell cycle phases. Using the dual-thymidine block technique it was possible to achieve over 70% synchronization after release of the block. It is also possible that no cell cycle differences existed in the cell line that they studied, which may have been non-neoplastic (it was described as of epithelial origin). Enhanced PDT sensitivity was observed in S-phase and G₂ of the cell cycle after a 1-h incubation with ALA, which may be due to the increased levels of PpIX in these cells. However, it may also be due to a differing sensitivity to reactive oxygen species at different phases of the cell cycle. Cellular sensitivity to radiotherapy (Sinclair and Morton, 1966), HPD-induced PDT (Christensen et al, 1981) and Photofrin II-induced PDT (Ma et al, 1995) varies with cell cycle phase. It has generally been found to be maximal in mitosis, late G₁ and early S-phase and least in early G₁ and late S-phase.

After a 4-h incubation period with ALA, the time usually used in clinical practice, the differential sensitivity was lost. The loss of differential PDT sensitivity, despite PpIX differences after a 4-h incubation with ALA, may reflect the increased PpIX in G₁ cells after 4 h that may have exceeded the PDT toxicity threshold. However, it is also possible that the loss of differential PDT sensitivity may be due to the timing of the light treatment protocol used. After a 1-h ALA incubation, cells are treated during early S-phase, which is relatively sensitive to damage, whereas with the 4-h incubation the cells are in late S-phase, which is relatively insensitive. Similarly, with the G₂ cells, after a 1-h incubation with ALA, the cell population may contain some insensitive late S-phase cells compared with the 4-h incubation when the population contains sensitive G₂ and M-phase cells. These differences may obscure any differential cytotoxicity at 4 h. It has also been suggested that intracellular photosensitizer distribution may vary with time and influence PDT sensitivity. With haematoporphyrin derivative and Photofrin, there is progressive shift in fluorescence from the plasma membrane after 3 h of drug incubation to the nuclear membrane and intracellular organelles by 7 and 24 h (Schneckenburger et al, 1988). With ALA-induced PpIX, these temporal alterations have not been observed, although only 15- and 42-h incubation times have been studied, which may have failed to detect any early changes (Gaulhier et al, 1995).

This study has also demonstrated that cell cycle phase-dependent differential sensitivity to PDT was no longer apparent when the light dose was increased to 1.5 J cm⁻², suggesting that appropriate light dosimetry may overcome this differential toxicity.

It is possible that the ability of cells to generate PpIX was affected by the synchronization protocol. Thymidine synchronization has been shown to influence the activity of DNase in HeLa cells leading to increased expression in S-phase cells compared with cells synchronized by selective mitotic detachment. However, the activities of other enzymes in this system were unaltered (Churchill and Studzinski, 1970). Another study observed no differences in the synthesis rates of various cellular proteins because of thymidine synchronization (Stein and Borun, 1972).

The dual thymidine block method was chosen for cell synchronization with this cell line as the other recognized methods of cell synchronization would not have been suitable for these experiments. A population of unsynchronized cells, labelled with both PpIX and a DNA-specific dye, were therefore studied to determine whether the thymidine synchronization protocol had any effect on PpIX levels between cell cycle phases. This study confirmed that PpIX levels are indeed lower in the G₁ phase cells than in the other two phases of the cell cycle after a 1-h incubation with ALA, although the amount of PpIX produced during S-phase was relatively lower in the unsynchronized cells. This cell cycle phase difference was also reproducible with several different cell types, both neoplastic (gastric cancer cells) and non-neoplastic (human dermal fibroblasts, data not shown). It should be noted however that with this technique the G₁ population will also include some early S-phase cells and the G₂ population will include some late S-phase cells that may interfere with some of the cell cycle phase-specific differences (Figure 4). Whether this reduced PpIX production is due to a proportion of the G₁ population representing the G₀ subgroup is not known. Exponentially growing immortalized tumour cell lines have few if any G₀ cells in their population, as suggested by the close correlation between the cell cycle time derived from BrdU pulse labelling and the population doubling time in the HT1197 cells. It is interesting to note that within the G₁ population (after a 1-h ALA incubation) there

is a broad range of PpIX fluorescence values, from 10 to 50 (arbitrary units), whereas the other two cycle phases have ranges from 30 to 50. The low fluorescent subpopulation of G₁ may be a PDT-resistant subgroup responsible for the increased percentage survival of G₁ cells after PDT.

In summary, it is possible that in a homogeneous population of tumour cells those cells in G₁ may be relatively insensitive to ALA-induced PDT (due to both decreased PpIX production and decreased PDT sensitivity) and thus survive treatment to provide foci of recurrence. However, there are many other confounding factors in the in vivo tumour microenvironment that may render cellular PpIX generation less efficient than in vitro, including pharmacokinetic bioavailability of ALA, poor tumour vascularity and hypoxia, which slows the rate of PpIX generation (L Wyld, unpublished data; Falk et al, 1959). It is also known that some of the toxicity of PDT may be due to microcirculatory collapse in the treated tissue, which may damage cells by inducing hypoxia (Reed et al, 1989), even if they have generated insufficient PpIX to suffer direct PDT toxicity. If PDT sensitivity, due to cell cycle variation, is a factor contributing to tumour recurrence, it could be overcome by the application either of adequate drug and light dosimetry or of a multiple-treatment regime to ensure cell cycle redistribution and tumour reoxygenation. This would allow relatively resistant G₁ cells to progress through the cell cycle to more sensitive cell cycle phases before administration of a second dose. At present, no data are available on whether cell cycle phase variation in PpIX generation or PDT sensitivity exists in vivo. Further in vivo studies are needed to validate these in vitro findings.

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