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Phototoxicity of 5-Aminolevulinic Acid in the HeLa Cell Line as an Indicative Measure of Photodynamic Effect After Topical Administration to Gynecological Lesions of Intraepithelial Form

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Purpose. The depth-resolved pattern of aminolevulinic acid (ALA) concentration in excised vaginal tissue was determined after *in vitro* application of an ALA-loaded bioadhesive patch. From this data, the tissue concentration of ALA achievable at a specified depth from the surface could be related to the concentration needed to elicit a photodynamic effect in a model gynecological tumor cell line (HeLa).
Methods. Excised vaginal tissue was mounted in a modified Franz diffusion cell and exposed to a water-soluble, ALA-loaded, bioadhesive patch. After a period of time, the tissue was cryostatically sectioned and the stratal concentration of radiolabeled ALA determined using scintillation spectroscopy. HeLa cells were cultured in media containing specific concentrations of ALA and exposed to standard photodynamic protocols of light exposure.
Results. An ALA concentration of 65.6 mM was achievable at 2.375 mm from the tissue surface after application of ALA-loaded patch. The photodynamic effectiveness of this concentration was demonstrated in HeLa with exposure to concentrations exceeding 1.0 mM ALA bringing about reductions in viable cell numbers by 90%. An enhancement of PpIX production using adjunctive EDTA over the clinically relevant 4 h application time interval was shown to be minimal in HeLa. Instead, PpIX production was more closely correlated with ALA concentration, with 100 mM ALA producing approximately 3100 ng PpIX mg^{-1} protein in the same time period.
Conclusions. Given that vaginal intraepithelial neoplasias can extend to 2.0 mm from the lesion surface, the ALA permeability derived from a bioadhesive patch is sufficient to induce photosensitization suitable for light induced destruction at deep sites of this type of lesion.

KEY WORDS: aminolevulinic acid; bioadhesive; HeLa; intraepithelial; photodynamic.

INTRODUCTION

Gynecological neoplasias of intraepithelial form represent pre-invasive and potentially malignant lesions of the squamous epithelium lining the vulva, vagina or cervix. They are the manifestation of a transepithelial infiltration by neoplastic cells, the extent of which is graded histologically, in that grade I neoplasias display abnormal cellular maturation that extends less than one-third the distance from the basal layer to the epithelial surface. In grade II, cellular maturation is abnormal up to two-thirds of the epithelial thickness and in grade III, there is a two thirds to full-thickness abnormality of cellular maturation (1). Grade III, or carcinoma *in situ*, is the most common presentation and, as with grades I and II, may be unifocal or, more commonly, multifocal (2).

Intraepithelial neoplasias constitute a significant health problem with an associated risk of progress to invasive disease. For example, vulval intraepithelial neoplasia (VIN), although uncommon, has undergone an increased incidence that has risen steadily from 1.8 to 2.8 per 100,000 from 1986 to 1998 (3). Around 25% of women with VIN have persistent or recurrent disease and, in symptomatic patients, the condition is associated with significant morbidity (4). Worryingly, the overall malignant potential of untreated VIN has been reported variously as being between 5% and 20% (4,5). Similarly, cervical intraepithelial neoplasia (CIN) has been linked to the incidence of cervical cancer, which in itself has been reported to result in 300,000 deaths per year, making it one of the largest causes of death in women worldwide (6). Vaginal intraepithelial neoplasia (VAIN) is usually seen coexisting with CIN in 1–6% of patients. It is almost always in the upper vagina and confluent with the cervical lesion (7).

Current treatment modalities for intraepithelial neoplasia are not without problems. Cold knife conization and loop electrosurgical excision are used extensively for eradication of CIN, but lead to deleterious outcomes, especially to the cervical stroma where scar stricture gives rise to problems related to infertility. Surgical intervention is favored for high-grade VIN and VAIN is treated using laser vaporization as the preferred method, as it minimizes damage to the bladder and bowel (8). Local administration of cytotoxic agents has been attempted, but concerns over the depth of drug penetration have arisen, especially for CIN, where crypts, often 2–3 mm below the colposcopically observable surface, can shelter neoplastic cells (9). There is little consensus as to an optimal drug-based treatment of VIN, with some reported use of topical dinitrochlorobenzene and 5-fluorouracil (10). Recurrence of gynecological neoplasias is common, regardless of the treatment modality used (11).

A promising alternative for the management of intraepithelial neoplasias is photodynamic therapy (PDT), which combines the action of visible light of appropriate wavelength and a photosensitizing drug to cause the destruction of selected cells (12,13). Aminolevulinic acid (ALA) is one of the most commonly used drugs for this purpose and is an example of the prodrug type, shown to be efficacious in early work by Kennedy *et al.* (14), who used it to treat basal cell carcinoma. It is an endogenous cellular component and is metabolized within the heme biosynthetic pathway to produce protoporphyrin IX (PpIX), a potent endogenous photosensitizer. This metabolic pathway is well regulated by feedback control arising from the presence of cellular concentrations of heme. As a consequence, heme biosynthesis is under such tight feedback control that only small amounts of PpIX are present in most cells at any given time. However, if excessive amounts of ALA are introduced midway into the cycle, presumably

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through drug delivery intervention, intermediates, such as PpIX, back-accumulate in cellular compartments in neoplastic cells. These cells are known to have a reduced capacity to metabolize PpIX to heme. The insertion of iron is performed by ferrochelatase and this step is believed to be a slow process, further adding to this accumulation of cellular PpIX. As a result, selective cells become photosensitized to the action of light. Accumulation of PpIX in the HeLa cell line has been demonstrated (15,16).

For gynecological PDT to be viable, it is important that the design of the drug delivery system enables it to locate firmly the lesion for a sufficient period of time. This has been difficult to achieve as most current strategies rely on applied creams or gels (10,11) or, less commonly, solutions (17). These are generally occluded under a foil or dressing immediately after application. This aids retention and enhances drug absorption, especially on keratinized surfaces, such as the vulva (2,4). Cervical caps may be used to aid retention of solutions in the cervix (18). In practice, occlusive dressings are poor at staying in place, particularly around the lower reproductive tract area, where shear forces dislodge the formulation as the patient moves. In addition, cervical caps may become displaced, leading to treatment failure. Ineffective exposure to the lesion will lead to an insufficient concentration of drug at the deepest region of the lesion.

This work describes the novel use of a bioadhesive patch for drug delivery purposes to gynecological intraepithelial neoplasias. Based on poly(methyl-vinylether/maleic anhydride), that has been shown to remain located firmly for up to 4 h to mucous and moist surfaces, such as the vaginal introitus and labia minora (19,20) and offers enhanced residence at lesion sites. For this reason, it is ideal for ALA-PDT, which often requires a minimum 4 h administration period. For effective photodynamic irradiation of intraepithelial neoplasia, it is important to achieve a therapeutic ALA concentration at the deepest site of the lesion. This work determines the ALA concentration at 2.375 mm from the surface of vaginal tissue as a model lesion. To ascertain if this concentration is indeed phototoxic, a model gynecological neoplastic cell line (HeLa) is used to determine the minimum threshold concentration of ALA that induces cell death. This is related to concentrations found in the excised vaginal tissue, thereby giving some indication if cell death on illumination is likely. This will give an indication if bioadhesive patch-based delivery of ALA to gynecological intraepithelial lesions is a viable alternative to surgical and laser based procedures.

MATERIALS AND METHODS

Materials

Gantrez AN-139, a copolymer of methylvinylether and maleic anhydride (PMVE/MA), was provided by ISP Co. Ltd. (Guildford, UK). Plastisol medical grade poly(vinyl chloride) (PVC) emulsion containing diethylphthalate as plasticizer, was provided by BASF Coatings Ltd. (Clwyd, UK). 5-Aminolevulinic acid hydrochloride and Porphin cream (20% w/w ALA in Unguentum Merck) were purchased from Crawford Pharmaceuticals (Milton Keynes, UK). Radiolabeled 5-aminolevulinic acid solution, 3.7 MBq ml^{-1}, was obtained from PerkinElmer Life Sciences (Beaconsfield, Bucks., UK). Tripropyleneglycol methyl ether (Dowanol TPM) and Protoporphyrin IX were purchased from Sigma Aldrich (Dorset, UK). All other chemicals were of analytical reagent grade. HeLa cell line ECACC No. 85060701 was obtained from the European Collection of Animal Cultures, PHLS Center for Applied Microbiology and Research (Porton Down, Wilts., UK).

Cell Culture Conditions

Cells of the line HeLa, derived from a human epidermoid cervical carcinoma, were used in this study. Cells were cultivated in Eagle's minimum essential medium (EMEM) containing 2 mM glutamine and 10% v/v fetal calf serum (FCS). The medium was supplemented with penicillin V (1.0 IU ml^{-1}) and streptomycin (1.0 $\mu g\ ml^{-1}$). Cells were routinely subcultured once weekly and maintained at 37°C and 5% CO_2 in a moist environment.

Incubation with ALA and Irradiation

Cells were harvested using 0.05% trypsin and 0.02% ethylene diamine tetra-acetic acid (EDTA), resuspended in fresh medium and seeded in a 96-well plate with black walls and a clear bottom. The initial cell concentration was 0.5×10^5 cells per well. Cells were then re-incubated for at least 15 h before further treatment. This gave rise to a cell layer in nearly exponential growth at the time of the experiments.

The pH of the ALA-containing medium was adjusted to pH 7.4 and used immediately after preparation, due to known instability of ALA at this pH in cell culture media (21). ALA was dissolved in RPMI 1640 serum-free medium to make a 100 mM stock solution. The medium was supplemented with penicillin V (1.0 IU ml^{-1}) and streptomycin (1.0 $\mu g\ ml^{-1}$). The pH of the solution was adjusted to pH 7.4 using 10 M sodium hydroxide solution. The stock solution was sterilized using appropriate filtration. Serial dilutions were performed under aseptic conditions, using serum-free RPMI 1640, to give ALA solutions of concentrations; 10.00 mM, 1.00 mM, 0.10 mM, and 0.01 mM.

The cell culture medium was removed from wells containing exponentially proliferating cells, which were then washed twice with 100 μl of serum-free RPMI 1640. Serum-free RPMI (100 μl), containing ALA, or ALA-free, was then added to each well. This medium is preferred because serum is known to cause efflux of PpIX from cultured cells (22). Cells were then re-incubated for 4 h before irradiation. This 4-h pre-irradiation interval was chosen to reflect the typical administration time of ALA-containing dosage forms used in clinical application. A separate 96-well plate was used for each ALA concentration. On each plate, 12 wells contained ALA and were irradiated, 12 wells contained ALA and were not irradiated, 12 wells did not contain ALA and were irradiated and 12 wells did not contain ALA and were not irradiated.

Wells were irradiated for 10 min through the clear lid of the plate using a Paterson Lamp (Phototherapeutics Ltd, Manchester, UK) at a wavelength of 635 nm. The optical dose delivered was 100 J cm^{-2}. Four wells were irradiated at a time, the remainder being shielded from light using black card. Following irradiation, the cell culture medium was removed and cells were washed twice in ice-cold phosphate-buffered saline pH 7.4 (PBS). The PBS was then replaced with RPMI

1640 containing 10% $^{v}/v$ FCS and the plate returned to the incubator for 20 h.

Cell Survival Analysis

Cell viability was tested by means of an MTT assay, a method based on the activity of mitochondrial dehydrogenases. This technique allows quantification of cell survival after cytotoxic insult by testing the enzymatic activity of mitochondria. It was deemed a suitable test for determination of cell viability after irradiation, as the porphyrin-loaded mitochondrion has been reported to be the first organelle to be affected by photo-induced damage (23). Reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide (MTT), to a purple, insoluble formazan derivative occurs in living, metabolically active cells.

A solution of MTT (10 μl), at a concentration of 10 mg ml^{-1}, was added to each well and the plate re-incubated at 37°C for 2 h. The medium was then removed and the cells washed with PBS. For cell lysis and dissolution of the formazan crystals formed, 200 μl of dimethyl sulphoxide was then added. Absorbance of each residue was determined at 540 nm using a Tecan multiwell plate-reading spectophotometer (Tecan UK Ltd, Reading, UK). Absorbance from the solution of cells incubated with ALA was divided by the absorption of the solution from control wells to calculate the fraction of surviving cells.

Protoporphyrin IX Formation

Protoporphyrin IX formation in cells incubated with varying concentrations of ALA was followed over a period of 24 h. Black-walled, clear-bottomed, 96 well plates were set up as described in 2.3, except that all 60 available wells had ALA-containing serum-free medium added. Again, separate plates were used for each ALA concentration and a blank plate, with only serum-free medium added, was used. Instead of irradiation being performed after 4 h of incubation, plates were removed from the incubator at regular, predefined, intervals. At each interval, the serum-free media was removed from five wells. The cells in these wells were then washed three times with cold PBS. Finally, the cells were brought into a solution of 0.5 M $HClO_4$ and 50% v/v methanol by means of a cell scraper. This solution has been found to monomerize porphyrins, so that their concentration can be reliably determined by fluorescence measurements (24). Plates were immediately returned to the incubator until the next time interval.

The Protoporphyrin IX (PpIX) content of the cells was measured fluorimetrically (Perkin Elmer LS55 Luminescence Spectrometer). The excitation wavelength was set at 408 nm and the fluorescence emission spectrum was scanned, using a long-pass cutoff filter (530 nm) to remove unwanted, scattered light. PpIX was quantified with respect to a standard curve (range 6 to 9000 ng ml^{-1}) and PpIX levels were expressed as ng μg^{-1} of protein, determined using the BCA assay described in "Protein Assay," below.

To assess the impact of iron chelation on PpIX production, EDTA (1.0 mM) was included in the ALA-containing media. All other conditions remained constant.

Protein Assay

Cells incubated, as described above, but not irradiated, were measured for protein content by the bicinchoninic acid (BCA) protein assay. Serum-free RPMI 1640 medium was carefully removed from the cell-containing wells and replaced with an equal volume of lysis buffer. This buffer contained 10 mM Tris BP, 10 mM EDTA and 0.2% v/v Triton X-100 detergent. The pH was pH 7.5. Cells, suspended in lysis buffer, were removed from the wells, pooled, and vortexed twice. After the first and second vortexing procedure, the sample was cooled on ice for 10 min. The sample was then repeatedly pumped up and down in a syringe with a 25-gauge needle to ensure completion of cell fragmentation. The resulting suspension was centrifuged at 3000 rpm for 5 min to pelletize the cellular debris. The supernatant was then collected

The BCA solution (200 μl) was added to samples of the supernatant in a 96-well plate and incubated for 30 min at 37°C. The samples were then read spectrophotometrically using the Cytofluor multiwell plate reader, set at a wavelength of 540 nm. The total protein content in each sample was calculated by comparing the means of three absorption values with a standard curve constructed from bovine serum albumin (BSA) dilutions (range 125 to 1000 μg ml^{-1}).

Microscopy Studies

Cells incubated, as described above, with or without ALA, were visualized using a Nikon Eclipse TE 300 microscope (Nikon Instruments, Melville, NY, USA). Cells were viewed under conventional light microscopy and using fluorescence microscopy with excitation at 425 ± 25 nm and emission at 650 ± 50 nm.

Patch Manufacture

Bioadhesive films evaluated in this study were prepared by a conventional casting technique using a 20% w/w PMVE/MA and 10% w/w TPM gel. PMVE/MA was added to ice-cooled water (reagent grade 1), stirred vigorously and heated to 95°C until a clear solution was formed. Upon cooling, the required amount of tripropylene glycol methyl ether (TPM) was added and the casting blend adjusted to a final weight with water. ALA (0.57 g) was dissolved directly into this aqueous blend (4.50 g) immediately prior to casting. Given the instability of ALA at elevated pH, no adjustment was required and was allowed to remain close to pH 2. Bioadhesive films were prepared by slowly casting the ALA-loaded gel into a pre-leveled mold (internal dimensions 30 mm by 50 mm), lined with a release liner to facilitate film removal This was placed in a constant air flow at 25°C for 24 h.

PVC backing films were prepared using a forced smear technique, where uncured polymer was knife-drawn over a glass surface to produce a film of approximately 100 μm thick. This was cured at 160°C, removed and applied to the exposed surface of the dry bioadhesive film, using gentle pressure to affirm attachment. Finished patches were removed by simply peeling the release liner, with attached film, off the base of the mould.

Evaluation of Static ALA Distribution in Vaginal Tissue

The distribution of drug in excised vaginal epithelial tissue after fixed time periods was determined using a Franz diffusion cell (Crown Glass Co. Inc., Sommerville, N.J., USA), as illustrated in Fig. 1. Tests were performed five times using water-jacketed cells, thermostatically maintained at

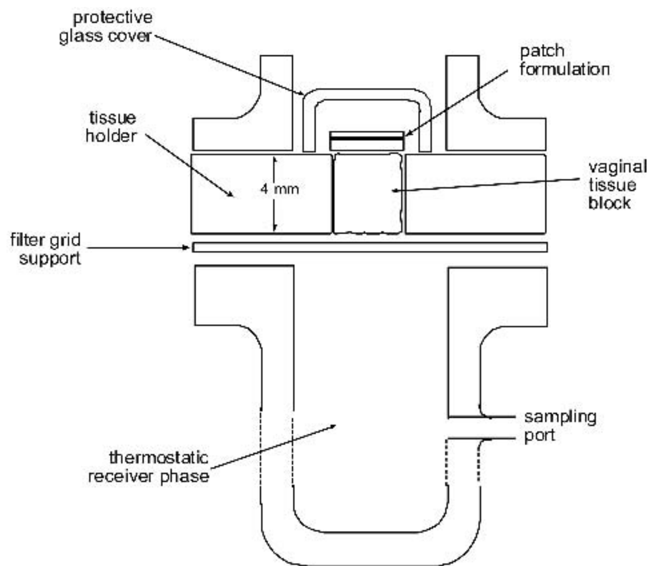

Fig. 1. Cross-sectional representation of diffusion cell apparatus used to hold vaginal tissue above a thermostatically controlled receiver phase. The patch or cream formulation is encapsulated under a protective glass cover that prevents tissue desiccation.

37°C. The receiver phase in each cell was stirred synchronously at 600 rpm.

Excised non-keratinizing vaginal tissue was recovered from routine reparative surgical procedures. This was flash-frozen immediately in a liquid nitrogen atmosphere and stored at –70°C. At the beginning of a penetration experiment, vaginal tissue was allowed to thaw slowly at 4°C and cut into cylinders, approximately 6 mm in diameter and 4 mm deep, using a proprietary dermatological punch. The tissue, epithelium face uppermost, was mounted in a purpose-built stainless steel washer, again 4 mm thick, with a circular hole, 5 mm in diameter, machined through its center. The tissue was supported on top of a stainless steel filter support grid that was placed across the top of the Franz cell reservoir. The reservoir was filled with approximately 10 ml of phosphate-buffered saline, pH 7.4, sufficient to bring the fluid level up to the grid and expel traces of air. Once the tissue had been mounted, a circular disc of bioadhesive patch, 5 mm in diameter and containing both radiolabeled and unlabeled ALA, was applied to the uppermost layer of the tissue slab, with the aid of a 5 μl drop of water. A glass cup, of diameter 8 mm, was placed over the assembly and secured with vacuum grease before the upper glass ring of the Franz cell was clamped in position. The patch and tissue were separated when the penetration time had elapsed, with the latter flash-frozen in a liquid nitrogen atmosphere prior to sectioning. By way of comparison, defined amounts of the proprietary Porphin cream (50 mg) were applied to the mounted tissue section. This was spiked extemporaneously with approximately 5.7×10^4 disintegrations per minute (dpm) of radiolabeled ALA. The experiment was performed in a similar fashion to that described above.

Microtome Procedure

Cylinders of frozen tissue, pre-exposed to both ALA-loaded formulations, were mounted on the stage of a cryostatic microtome (Leica Microsystems, Nussloch, Germany) using tissue embedding fluid. The microtome environment and stage operated at –25°C. Tissue cylinders were positioned so that their upper surfaces, to which patches had been attached, were parallel to the slicing motion of the blade. Slice thickness was set at 50 μm. Five consecutive slices were taken and placed into a scintillation vial. This procedure was repeated ten times for each tissue sample, such that the total depth of slicing into the tissue was 2.5 mm.

Determination of Radiolabeled ALA

Tissue slices containing radiolabeled ALA were dissolved in 1 ml NCS-II overnight at 37°C. Scintillation cocktail (10 ml) was added to each vial and mixed. Vials were stored in darkness for 2 h prior to analysis to reduce chemiluminescence to less than 1% in respect of the total count. Samples were counted for 10 min, with conversion to dpm achieved against quench correction curves. Mean dpm values from 3 replicate experiments were converted to mean ALA concentrations and related to penetration depth.

Statistical Analysis

The influences of different ALA concentrations and irradiation on the number of surviving HeLa cells were analyzed using the Wilcoxon signed-rank test. This test was also used to analyze the influences of increasing ALA concentrations and addition of EDTA on the amount of Protoporphyrin IX produced by cells. Comparison between cream and patch formulations, in terms of ALA penetration, was made using the Mann-Whitney *U* test. The Kruskal-Wallis test was used to analyze the effects of increasing application times on ALA penetration. In all cases, $p < 0.05$ denoted significance.

RESULTS

Figure 2 shows the influences of increasing concentrations of ALA in the absence of irradiation on the survival rates in wells containing 0.5×10^5 viable HeLa cells. The numbers of viable cells, as determined using the MTT assay,

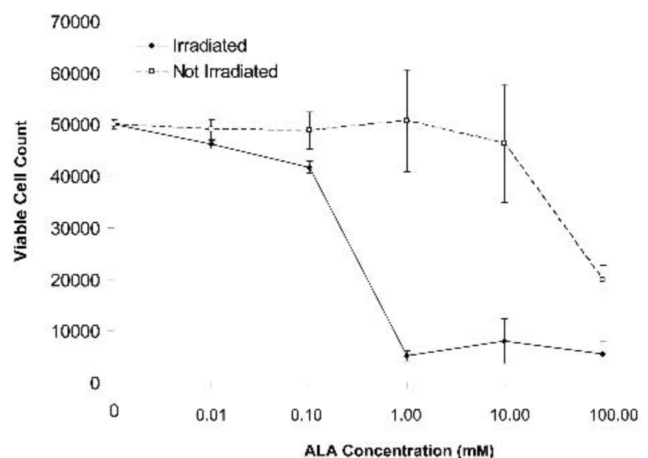

Fig. 2. Influences of increasing ALA concentrations and irradiation with 100 J cm^{-2} of red light (635 nm) on the numbers of surviving HeLa cells in wells in 96-well plates originally containing approximately 50,000 viable cells. Cells were incubated with ALA for 4 h before irradiation (mean ± SD, n = 12).

was shown to remain significantly unchanged ($p = 0.7353$) as the ALA concentration was increased to 10 mM and irradiated was not used. However, incubating cells in 100 mM ALA caused a significant ($p = 0.0180$) reduction in the number of viable cells, such that only 35% of the original viable cells remained.

Exposure to light alone was shown to be an innocuous process, as demonstrated by no significant reductions ($p = 0.7150$) in the viable cell count, when compared to control incubations free of both ALA and light radiation. Incubation of cells with 0.01 mM ALA for 4 h, followed by irradiation, caused a significant decrease ($p = 0.0218$) in the number of viable cells, as did incubation with 0.1 mM ALA ($p = 0.0051$). Indeed, increasing the ALA concentration to 1.0 mM caused a profound decrease in the number of viable cells following irradiation, such that only around 10% of the original viable population remained. Further increasing the ALA concentration to 100 mM did not cause any further significant cell death ($p = 0.2393$). Even at this elevated concentration, the effect of light was still shown to augment cell death ($p = 0.0173$) as demonstrated by a significantly reduced viability compared to those incubated with 100 mM ALA, but not irradiated.

Figure 3 shows the effect of increasing concentrations of ALA in the presence of 1.0 mM EDTA on the amount on protoporphyrin IX (PpIX) formed after 4 h of incubation. This particular time period was chosen because dosage forms used in clinical PDT are frequently left *in situ* for this length of time. EDTA has been shown to increase PpIX formation by way of Fe^{3+} sequestration and has been a popular approach to increase PpIX formation. Increasing ALA concentration from 0.0 mM to 0.01 mM ($p = 0.6858$) or to 0.1 mM ($p = 0.2733$) had no significant effect on the amount of PpIX formed. However, increasing the ALA concentration from 0.0 mM to 1.0 mM caused a significant increase ($p = 0.0051$) in the amount of PpIX formed after 4 h incubation. Further increasing the ALA concentration caused a further significant increase in the amount of PpIX formed. The amount of PpIX formed after 4 h incubation in 1.0 mM ALA-containing media was approximately 900 ng mg^{-1} protein, while the amount formed after 4 h incubation in 100 mM ALA was approximately 3100 ng mg^{-1} protein. Apart from cells incubated in 100 mM ALA ($p = 0.0051$), the presence or absence of EDTA was shown to produce no significant difference in the

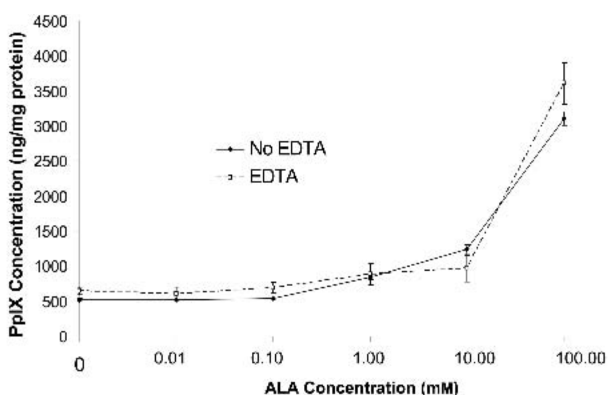

Fig. 3. The concentration dependence of protoporphyrin IX production in HeLa cells after exposure to increasing amounts of ALA in either the presence or absence of 1.0 mM EDTA. The incubation time used was 4 h (mean ± SD, n = 5).

amounts of PpIX formed at any particular ALA concentration.

Figure 4a shows the time related accumulative PpIX concentration in HeLa cells when periods of time are considered beyond the clinically relevant 4 h application interval. The effect of 0.01 mM ALA and 1.0 mM EDTA on PpIX formation in untreated cells and those incubated in 0.01 mM ALA alone did not change significantly over 24 h. However, after 24 h, cells incubated with 1.0 mM EDTA alone had produced an amount of PpIX that was significantly greater than wholly untreated cells ($p = 0.0051$). Cells incubated in 0.01 mM ALA containing 1.0 mM EDTA produced around 2100 ng PpIX mg^{-1} protein after 24 h incubation. This was significantly greater than that produced by both untreated cells ($p = 0.0051$) and those treated with 1.0 mM EDTA alone ($p = 0.0051$). Figure 4b shows the 24-h PpIX accumulation profiles when ALA concentrations are incremented upwards (0.1 mM or 1.0 mM) with, or without, 1.0 mM EDTA. Over the first 7 h, PpIX accumulated at a steady rate in treatment groups using the higher ALA concentration. The treatment group using 0.1 mM ALA and no EDTA displayed no discernable rise in PpIX levels over this time interval. Unexpectedly, cells incubated in 1.0 mM ALA, containing 1.0 mM EDTA, produced significantly less PpIX over the first 7 h than cells treated with either 1mM ALA alone ($p = 0.0129$) or with 1.0 mM EDTA in 0.1 mM ALA ($p = 0.0051$). However, after 24 h, cells incubated in 1.0 mM ALA, containing EDTA, had produced approximately 2700 ng PpIX mg^{-1} protein, which was significantly greater than that produced by cells subjected to either of these other treatments. Cells incubated in 0.1 mM ALA alone produced the least PpIX over the 24-h period. Finally, Fig. 4c shows the 24-h PpIX accumulation profiles in either 10 mM or 100 mM ALA with, or without, 1.0 mM EDTA. The amount of PpIX produced by cells incubated with 100 mM ALA alone increased steadily over the first 7 h, at which point approximately 5200 ng PpIX mg^{-1} protein had been produced. Cells incubated with 10 mM ALA alone also steadily accumulated PpIX over the first 7 h. However, the amount of PpIX produced was significantly less than that produced by cells incubated with 100 mM ALA alone ($p = 0.0051$). After 7 h incubation, EDTA inclusion did not significantly increase the amount of PpIX produced by cells incubated with10 mM ALA ($p = 0.0077$). Addition of 1.0 mM EDTA significantly increased the amount of PpIX produced by cells incubated in 100 mM ALA over the first 7 h ($p = 0.0051$). However, iron chelation had no significant effect upon consideration of the complete 24-h incubation duration ($p = 0.0663$). In contrast, addition of EDTA significantly increased the amount of PpIX produced by cells incubated in 10 mM ALA over 24 h ($p = 0.0475$). It is clear form Fig. 5 that cells incubated in 10 mM ALA produced significantly less PpIX over 24 h than those incubated in 100 mM ALA, regardless of EDTA addition.

Figure 5 shows HeLa cells viewed under conventional light and fluorescence microscopy. ALA exposure (4 h, 10 mM) has not resulted in changes to the confluent growth of the HeLa cells (Fig. 5a). The presence of PpIX is seen clearly in Fig. 5b, producing the characteristic red emission when illuminated under near UV conditions. In the absence of ALA, this red colouration is considerably less (Fig. 5c).

Table I presents the concentration of ALA found at 2.375 mm from the tissue surface. Increasing the application

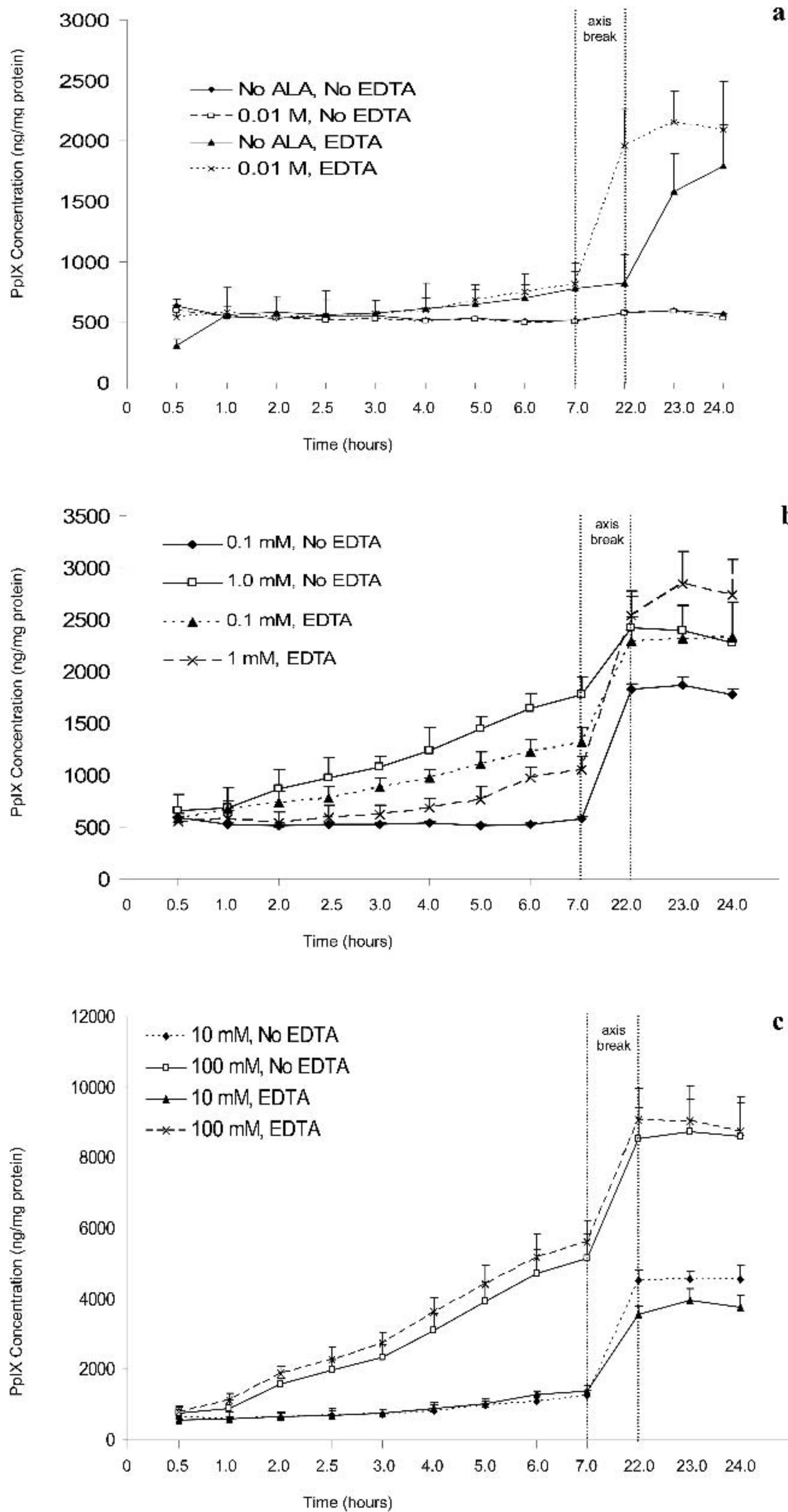

Fig. 4. PpIX production in HeLa cells over a 24-h period in the presence of 1 mM EDTA and increasing concentrations of ALA, (a) 0.00 mM to 0.01 mM, (b) 0.10 mM to 1.00 mM, and (c) 10 mM to 100 mM. (Mean, n = 5. For clarity, only positive error bars shown.)

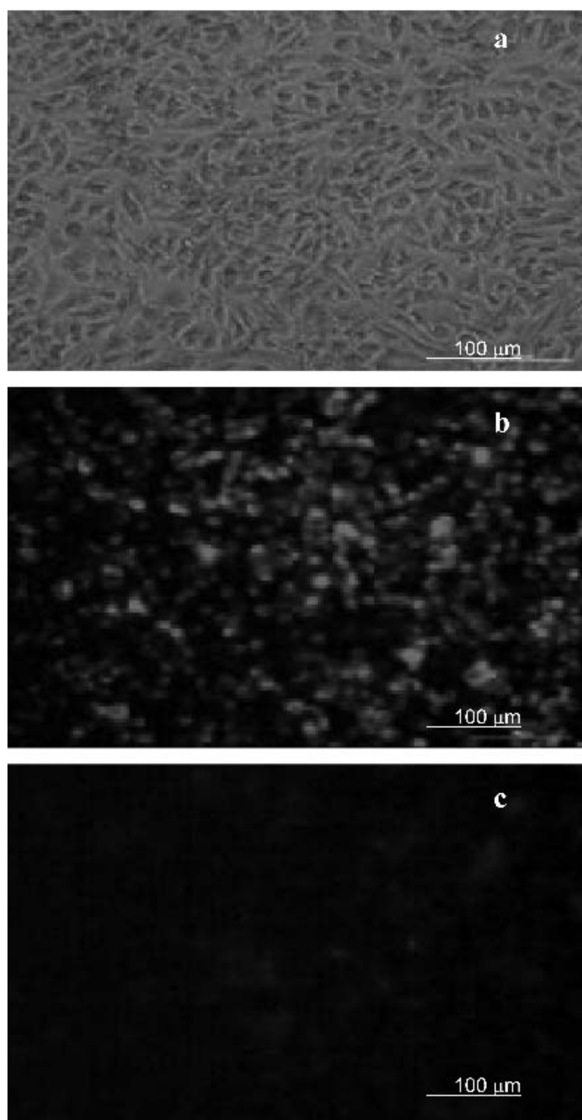

Fig. 5. Light micrograph of (a) HeLa cells incubated for 4 h in 1.0 mM ALA, (b) fluorescence micrograph of HeLa cells incubated for 4 h in 1.0 mM ALA, and (c) fluorescence micrograph of HeLa cells incubated for 4 h without ALA. Excitation was at 425 ± 25 nm and emission was at 650 ± 50 nm in both cases.

time significantly increased the ALA found at this depth. Application of Porphin cream for 4 h led to an ALA concentration at 2.375 mm, which was not significantly different ($p = 0.5127$) to that produced by the patch containing 38 mg ALA cm^{-2}. This patch was designed to deliver the same amount of drug per unit area and was applied also for 4 h. Similarly, the ALA concentrations at depths of 2.375 mm in tissue after 1 ($p = 0.5127$) or 2 ($p = 0.8273$) hours of cream application were not significantly different to those after 1 or 2 h of application of the 38 mg cm^{-2} patch. The cummulative mass of ALA determined in the tissue is also shown expressed as a percentage of the total available in the applied dosage system. This shows that less than 20% of the total ALA available is delivered to the tissue in a 4 h duration.

DISCUSSION

It is well established that ALA-induced PpIX is an effective cytotoxic photosensitizer for the treatment of neoplasias (14,25). Lesions of a superficial nature can be treated using a topically applied dosage form that is loaded with a photosensitizer, such as ALA. Effective eradication then depends on achieving and exceeding simultaneous thresholds of dosimetry of light and concentration of ALA at the most distant regions of the tumor. If either is not fulfilled, then the clinical outcome will be one of PDT failure and higher than expected rates of reoccurrence. For these reasons, it is important that the concentration achieved at these deep sites after topical application exceeds that shown to exert a phototoxic effect. Perhaps the simplest way to achieve some measure of this minimum phototoxic concentration is to expose a defined cell line to media containing known concentrations of photosensitizer. For example, incubating fibrosarcoma cells with 0.6 mM ALA for 4 h, followed by irradiation (0–15 J cm^{-2}) with red light (635 nm), reduced the number of viable cells to less than 10% of their original number (23). To compliment this information, it is important to determine the achieved concentration at a defined distance from the surface of the lesion, once the topical dosage form has been removed. A simple judgement can then be made to see if the tissue concentration exceeds that needed to bring about cell death.

In this study, healthy vaginal tissue was used as a model for an intraepithelial neoplasia. Vaginal tissue is easily recoverable form reparative surgical procedures, whereas excised cervical and vulval material is considerably more difficult to obtain in appropriate quantities. The length of time most frequently used for ALA administration in clinical PDT is 4 h. As shown in Table I, the permeation resulting from 4 h application of bioadhesive patch containing 38.0 mg ALA cm^{-2} was sufficient to produce an ALA concentration of approximately 11 mg cm^{-3} at a mean depth of 2.375 mm in vaginal tissue. Assuming vaginal tissue has a specific gravity close to that of water, this approximates to an ALA concentration of 65.6 mM at 2.375 mm. This gives an important estimation of the ALA concentration that could be achieved at the deepest VAIN sites, given two important caveats. First, that the maximum depth of such lesions is in the region of 2.5 mm, which has been reported (26) and, second, the ALA permeability of a VAIN lesion resembles that of the normal corresponding squamous epithelium.

HeLa was used as a neoplastic model cell line of gynecological origin in this work. It is an epitheloid cell line and, therefore, closely approximates to histologic form of vaginal intraepithelial neoplasias. Ideally, a primary culture from an actual vaginal lesion would improve the experimental design, but the lack of available transformed vaginal cells would require the establishment of a primary culture from biopsy. Given the sensitivities of these types of culture to environmental effects, alternations in cell numbers due to phototoxic effects alone may be more difficult to ascertain. The robustness of the HeLa line makes it a reproducible working model. In addition, the pH of the culture media was not controlled for the purposes of maintaining ALA stability throughout the duration of the experiment. This approach has been adopted by other workers (23) and used in this work primarily in an attempt to mimic *in vivo* conditions, where ALA is released into local tissue conditions and where pH is expected to be close to that of physiologic. Arguably, increased ALA degradation is inevitable, but cellular quiescence, as discussed below, may make the interpretation of PpIX levels difficult.

Table I. Penetration Properties of 5-Aminolevulinic Acid (ALA) into Vaginal Epithelial Tissue

Formulation	Application time (h)	Percentage of total ALA loading released into tissue (±SD), n = 3	ALA concentration at 2.375 mm (mg cm^{-3}) (± SD), n = 3
Porphin cream	1	8.09 ± 0.74	3.18 ± 0.14
Porphin cream	2	7.93 ± 0.86	5.65 ± 1.52
Porphin cream	4	15.28 ± 4.06	9.42 ± 4.41
38 mg cm^{-2} patch	1	8.50 ± 2.80	2.31 ± 1.16
38 mg cm^{-2} patch	2	18.53 ± 3.75	6.00 ± 2.56
38 mg cm^{-2} patch	4	17.65 ± 3.00	10.93 ± 0.24

The effect of light by itself did not significantly alter the cell viability, as shown in Fig. 2. Upon irradiation and exposure to media containing ALA, reductions in the numbers of viable cells were observed, with concentrations in excess of 1.0 mM ALA needed to reduce the viable population to 10%. This can be compared to a reduction of 90% in the number of viable WiDr (recto-sigmoid colon adenocarcinoma) cells after 4 h incubation with 0.05 mM ALA and irradiation with a light dose of 3.6 J cm^{-2} at 635 nm (27). However, 40 min irradiation was required at this low ALA concentration. Other work has shown the optimal ALA concentration for successful PDT of human cervical carcinoma cells to be 10 mM (28). However, above this concentration, the drug itself became toxic to cells without the intervention of irradiation. In this study, only an ALA concentration of 100 mM caused a toxic effect to be observed in the absence of irradiation. It is now clear that an ALA concentration of 65.6 mM recovered in tissue is more than sufficient to illicit a phototherapeutic effect. However, such a high concentration may cause problems in itself. Work using fibrosarcoma cells showed that the optimal phototoxic effect was observed at an extracellular pH value of pH 7.4 (23). Values above and below pH 7.4 reduced the effectiveness of PDT. This was in contrast to the results obtained after photo-irradiation of bladder cancer cells, where maximum phototoxicity was found at pH 7.0 and pH 6.5 and reduced PDT efficacy at pH 7.4 and pH 5.5 (29). It is known that ALA uptake is maximal around pH 5.0 (30). It is probable that a reduced extracellular pH induced by high local concentrations of ALA may induce cellular quiescence. This effect has been shown by other workers and may reduce the effectiveness of PDT by retarding the metabolic conversion of ALA to PpIX (31,32). The pH of a 65.6 mM solution of ALA in distilled water is 3.2. The pH of 65.6 mM ALA in a vaginal tissue homogenate is marginally higher at 4.0, presumably due to the buffering capacity of cellular proteins. There is a distinct danger that achieving effective drug delivery of ALA is balanced by an attenuation of the metabolic capacity of enzymes in the heme biosynthetic pathway arising from reduced pH.

Comparison of the fluorescence spectra of cell samples and a PpIX standard, in the same medium, showed the fluorescing compound to be PpIX. After 4 h incubation, the amount of PpIX produced by HeLa cells was strongly dependent on the ALA concentration. Cells incubated in media containing ALA concentrations lower than 1.0 mM did not produce any more PpIX than the untreated control and this may reflect their reduced response to irradiation. Cells incubated with 1.0 mM ALA, for 4 h, showed strong red PpIX fluorescence, in contrast to untreated cells, as shown in Fig. 5. Cells incubated in 1.0 mM ALA produced approximately 900 ng PpIX mg^{-1} protein. This is of the same order as the amount of the photosensitizer formed in WiDr cells (27,33) and in bladder cancer cells (29), incubated for 4 h in 1.0 mM ALA.

If excessive amounts of ALA reduce PpIX production, then adopting a strategy to enhance the effectiveness of lesser amounts may seem feasible (23). One such approach uses EDTA. This membrane impermeable chelator has affinity for several cations, of which that for Fe^{3+} is notably high. It has been postulated that chelation of intracellular iron elevates PpIX levels by reducing its rate of ferrochelatase-mediated conversion to heme. After 4 h incubation with 1.0 mM EDTA, only cells incubated in 100 mM ALA accumulated significantly more PpIX than cells incubated in ALA alone. It is doubtful that, over a typical application time of 4 h, EDTA will have a beneficial effect on topical treatments of intraepithelial neoplasias, as shown by the PpIX levels in Fig. 4b at that time point.

The amount of PpIX formed by HeLa cells over 24 h increased with increasing ALA concentration. Cells incubated in 1.0 mM ALA produced around 2300 ng PpIX mg^{-1} of protein after 24 h incubation. Similar yields of photosensitizer were detected during incubation studies with WiDr cells in 1.0 mM ALA for 24 h (34). Addition of 1.0 mM EDTA significantly increased the amount of PpIX produced over 24 h by cells incubated in media with ALA concentrations of 10 mM or less. However, EDTA could not significantly increase the amount of PpIX produced over 24 h by cells incubated in 100 mM ALA. This observation may be attributed to the tendency of ferrochelatase to become saturated when PpIX levels are high, due to the prolonged exposure to large amounts of ALA (35).

In conclusion, a novel drug-loaded bioadhesive patch applied to the surface of excised vaginal tissue produced an ALA concentration of 65.6 mM at 2.375 mm from the epithelial surface. This concentration was shown to exceed that needed for photoinduced cell death in the HeLa cell line by a considerable margin. As most vaginal intraepithelial neoplasias do not extend down deeper than this, then the bioadhesive patch can act as an effective drug delivery system for the PDT of lesions of this nature. The simultaneous use of EDTA was shown to be of little benefit after 4 h use, an important time point in clinically-based PDT. However, some benefit from EDTA was observed if the duration of application was allowed to extend to 24 h. The patch was shown to match the currently used cream formulation in respect of the amount of

ALA that penetrated into the tissue, but offers the distinct advantage in being able to remain in place for extended periods of time without the need for occlusion.

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