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Research paper

In vitro phototoxicity of 5-aminolevulinic acid and its methyl ester and the influence of barrier properties on their release from a bioadhesive patch

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Abstract

Topical administration of excess exogenous 5-aminolevulinic acid (ALA) leads to selective accumulation of the potent photosensitiser protoporphyrin IX (PpIX) in neoplastic cells, which can then be destroyed by irradiation with visible light. Due to its hydrophilicity, ALA penetrates deep lesions, such as nodular basal cell carcinomas (BCCs) poorly. As a result, more lipophilic esters of ALA have been employed to improve tissue penetration. In this study, the in vitro release of ALA and M-ALA from proprietary creams and novel patch-based systems across normal *stratum corneum* and a model membrane designed to mimic the abnormal *stratum corneum* overlying neoplastic skin lesions were investigated. Receiver compartment drug concentrations were compared with the concentrations of each drug producing high levels of PpIX production and subsequent light-induced kill in a model neoplastic cell line (LOX). LOX cells were found to be quite resistant to ALA- and M-ALA-induced phototoxicity. However, drug concentrations achieved in receiver compartments were comparable to those required to induce high levels of cell death upon irradiation in cell lines reported in the literature. Patches released significantly less drug across normal *stratum corneum* and significantly more across the model membrane. This is of major significance since the selectivity of PDT for neoplastic lesions will be further enhanced by the delivery system. ALA/M-ALA will only be delivered in significant amounts to the abnormal tissue. PpIX will only then accumulate in the neoplastic cells and the normal surrounding tissue will be unharmed upon irradiation.

Keywords: Photodynamic therapy; 5-Aminolevulinic acid; *Stratum corneum*; Patches; Drug delivery; Bioadhesion

1. Introduction

Photodynamic therapy (PDT) based on topical application of 5-aminolevulinic acid (ALA) has been shown to achieve high clearance rates when used in the treatment of superficial skin lesions, such as basal cell carcinoma, Bowen's disease and actinic keratosis [1]. ALA, a naturally occurring precursor in the biosynthetic pathway of haem, is now the most commonly used drug in modern dermatological PDT. Topical administration of excess exogenous ALA leads to selective accumulation of the potent photosensitiser protoporphyrin IX (PpIX) in neoplastic cells which can then be destroyed by irradiation with visible light. Excellent tissue preservation and lack of scarring are noted advantages over conventional surgical treatment options for such lesions. However, deep lesions, such as nodular basal cell carcinomas, or those with overlying keratinous debris are reported as being resistant to ALA PDT [1,2]. Such treatment failures have been attributed to the water solubility of the drug preventing its deep penetration into tissue [1]. Consequently, more lipophilic ALA derivatives have been produced with the aim of improving drug penetration and subsequent treatment success rates. The

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most commonly investigated ALA derivative has been its methyl ester (M-ALA). Improved clearance rates of nodular basal cell carcinomas have been reported after topical application of M-ALA [3].

ALA and M-ALA are typically delivered to surface lesions using topically applied creams which are covered with occlusive dressings to aid retention at the site and enhance drug absorption [4]. However, great variability in the amount of such creams applied per unit area has been reported [1,5–7]. Application of occlusive dressings over the applied creams leads to smudging and spreading of the cream away from the site of application in an irreproducible fashion, adding further uncertainty to the technique. Consequently, comparison of the results of different studies is difficult. Clearly there is a need for a unit dosage form for use in PDT based on the topical application of ALA or its derivatives, such as M-ALA. Use of such a system would eliminate the inter-clinician variability seen at present and would allow accurate critical comparisons of different studies to be made. An ideal dosage form would be self-adhesive and backed with an occlusive material, thereby negating the need for a covering dressing and simplifying treatment.

This paper describes the design of a bioadhesive patch intended as a topical delivery system for ALA or M-ALA. Drug release from the system across a model membrane and excised *stratum corneum* is evaluated and compared in each case with that from the relevant proprietary cream. Treatment success depends on attainment of a concentration of ALA or M-ALA sufficient to yield photosensitising concentrations of PpIX in viable neoplastic tissue. Therefore, the concentrations of ALA or M-ALA produced on the receptor side of a model membrane and excised *stratum corneum* are compared with the concentrations shown to be phototoxic to a model cell line derived from a skin neoplasm.

2. Materials and methods

2.1. Chemicals

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) emulsion containing diethylphthalate as plasticiser was provided by BASF Coatings Ltd., Clwyd, UK. 5-Aminolevulinic acid, hydrochloride salt and Porphin® cream were purchased from Crawford Pharmaceuticals, Milton Keynes, UK. Triton® X-100 was purchased from Amersham Biosciences, Bucks, UK. 5-Aminolevulinic acid, methyl ester, hydrochloride salt, trypsin type III and tripropylene glycol methyl ether (Dowanol™ TPM), Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, non-essential amino acids, sodium pyruvate, cell dissociation solution, phosphate buffered saline tablets, ethylenediaminetetraacetic acid (EDTA) and trypsin/EDTA solution, trypan blue solution (0.4%), dimethyl sulphoxide and L-glutamine were purchased from Sigma–Aldrich, Dorset, UK. Foetal calf serum (FCS) was purchased from Gibco Ltd. (Paisley, Scotland). Nu-serum was purchased from BD Biosciences (Bedford, MA). Penicillin/streptomycin solution was supplied by Invitrogen Life Technologies Ltd. (Paisley, Scotland). Metvix® cream was provided by Galderma UK Ltd., Hertfordshire, UK. All other chemicals were of analytical reagent grade.

2.2. Membranes for drug release

Cuprophan® dialysis membrane sheets, molecular weight cut-off 10,000 Da, were obtained from Medicell International, London, UK. Porcine skin is a good model for human skin with regard to hair sparseness, presence of subcutaneous fat, epidermal proliferation, and both the orientation and distribution of blood vessels [8,9]. Stillborn piglets were obtained from a local abattoir and abdominal full-thickness skin was immediately excised. Subcutaneous fat and connective tissue were removed using forceps after immersion in water at 60 °C for 2 min [10,11]. The *stratum corneum* was separated from the epidermis after floating on a solution of trypsin type III (0.1% w/v) and sodium bicarbonate (0.5% w/v) at room temperature as described by Bentley et al. [12]. The enzyme digests the nucleated epidermal tissue, enabling the remnants to be removed by gentle rubbing with cotton wool. The *stratum corneum* sheets were rinsed repeatedly with distilled water, gently pressed between tissue paper, spread on filter paper and subsequently dried by storage in a desiccator over silica gel for a maximum of 2 weeks before use.

2.3. Cell line

Cells of the line LOX, derived from a human amelanotic melanoma, were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 medium supplemented with 10% FCS, 5% Nu-serum, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, penicillin (1.0 IU ml^{-1}) and streptomycin (1.0 μg ml^{-1}). Cells were routinely subcultured once weekly and maintained at 37 °C and 5% CO_2 in a moist environment. The cell line was kindly provided by Prof. Øystein Fodstad, Norwegian Radium Hospital, Montebello, Oslo, Norway.

2.4. Patch manufacture

Bioadhesive patches evaluated in this study were prepared by a conventional casting technique [13] 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. Appropriate amounts of ALA and M-ALA were dissolved

directly into defined volumes of this aqueous blend immediately prior to casting.

Patches containing ALA loadings of 19, 38 and 50 mg cm^{-2} were prepared, while patches containing M-ALA loadings of 15.2, 30.4 and 40 mg cm^{-2} were also made. Determination of approximate loadings of ALA and M-ALA to be included in bioadhesive patches was by consultation with a clinician experienced in the use of topical creams in PDT. It was decided to load each square centimetre of the patch with an equivalent dose of ALA or M-ALA to that contained in the amounts of proprietary creams typically applied per square centimetre to neoplastic lesions.

A cream (Unguentum Merck®) was applied, in the thickness used clinically, to each of 25 cm^2, ruled out on the back of a gloved hand. Each cm^2 was individually cleared of cream using a microspatula and each aliquot of cream weighed. As Porphin® contains 20% w/w ALA and Metvix® contains 16% w/w M-ALA, the mean ALA and M-ALA doses per cm^2 were determined by calculation. This estimation of drug loading was then used as a starting point in the patch design process.

Given the instability of ALA and its esters at elevated pH [14], no adjustment was required and the pH of the cast gels was allowed to remain close to pH 2. Bioadhesive films were prepared by slowly casting the drug-loaded gel into a pre-levelled mould (internal dimensions 30 × 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.

2.5. Drug release studies

The release of ALA from patch formulations was investigated using the modified Franz cell apparatus shown in Fig. 1. The orifice diameter in both donor and receptor compartments was 15 mm. Receptor compartment volumes, approximately 10 ml, were exactly determined by triplicate measurements of the weights of water they could accommodate. Account was taken for the volumes occupied by magnetic stirring bars. Compartment temperatures were kept constant at 37 °C by recirculating water from a thermostatically controlled bath. The receptor phase was 0.1 M borate buffer, pH 5 (*Pharmacopoeia helvetica*). This buffer was used since it was shown to maintain ALA stability at a high concentration (8 mg ml^{-1}) at temperatures up to 37 °C for periods of up to 6 h [15]. The buffer was degassed prior to use by vacuum filtration through a HPLC filter. Continuous stirring was provided by Teflon-coated stirring bars, rotating at 600 rpm. Stainless steel

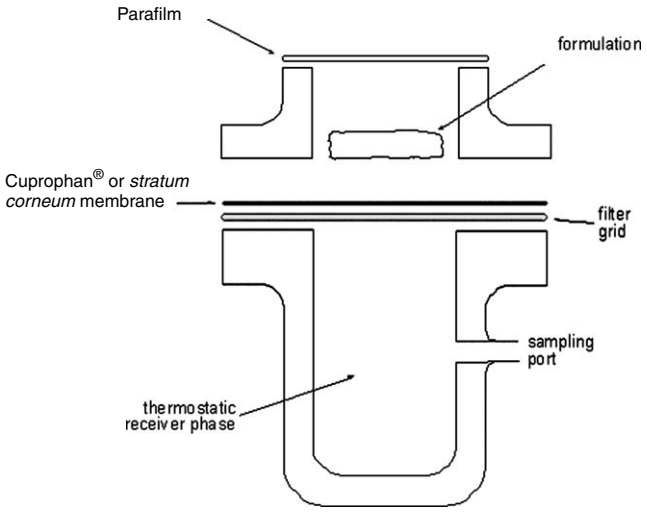

Fig. 1. Apparatus used in drug release experiments investigating the release of ALA and M-ALA from patches and creams across Cuprophan® and *stratum corneum* membranes.

filter support grids were used to support Cuprophan® or *stratum corneum* membranes. The membranes and support grids were sandwiched between the donor and receptor compartments. The thickness of each individual *stratum corneum* membrane was determined using a digital micrometer (Mitutoyo Corporation, Japan) prior to use. High vacuum grease and spring clips were used to hold the entire assembly together. The donor compartments were covered with laboratory film (Parafilm®).

Release from ALA- and M-ALA-loaded patches was investigated by first cutting circular discs from 3 × 5 cm patches using a sharp circular cork borer of inside diameter 1.5 cm. The bioadhesive surfaces of these discs were attached to the Cuprophan® or *stratum corneum* membranes in the donor compartments using 10 μl of deionised water. Using a long needle, samples (0.25 ml) were removed from the receptor compartment at defined time intervals (5, 10, 15, 30, 60, 120, 180, 240, 300, and 360 min). This volume was immediately replaced using blank, pre-warmed buffer. Samples removed were diluted to 5 ml with buffer and analysed by HPLC, as described below. Results were reported as means (±SD) of five replicates. The release of ALA and M-ALA from the proprietary Porphin® and Metvix® creams, respectively, was also investigated in this way. The creams were applied, in the thickness used clinically, to the membranes in the donor compartments. Again, ALA and M-ALA were determined using HPLC and results reported as means (±SD) of five replicates.

2.6. Determination of ALA and M-ALA

ALA and M-ALA released from patches and creams were quantified using HPLC, employing pre-column derivatisation with acetyl acetone and formaldehyde and fluorescence detection, as described by Oishi et al. [16]. In brief,

acetyl acetone reagent was prepared by mixing 15 ml acetyl acetone, 10 ml ethanol and 75 ml distilled water. Formaldehyde solution (10% w/w) was made by a 3.7-fold dilution of the chemical reagent (37% w/w) with distilled water and stored in the dark.

Derivatisation was based on a modification of the Hantzsch reaction, in which amine compounds react with acetyl acetone and formaldehyde. To a HPLC vial, 3.5 ml acetyl acetone reagent, 50 μl of sample and 0.45 ml of 10% w/w formaldehyde solution were added and mixed on a vortex mixer for approximately 5 s. This mixture was heated for 10 min at 100 °C. The vial was then cooled in an ice bath.

The solution containing ALA-acetyl acetone/formaldehyde reagent derivative was injected (10 μl, Waters WISP 712 autoinjector with refrigerated sample compartment, Waters associates, Harrow, UK) onto a Spherisorb® column (250 × 4.6 mm, C_{18} ODS2 with 5 μm packing, Waters associates, Harrow, UK) equipped with a Spherisorb® guard column (10 × 4.6 mm, C_{18} ODS2 with 5 μm packing). The mobile phase was 49.5% methanol/49.5% water/1% glacial acetic acid v/v/v. The flow rate was 1.5 ml min^{-1} (LKB Bromma 2150 liquid chromatography pump, LKB Company, Uppala, Sweden). Detection was by fluorescence with excitation at 370 nm and emission at 460 nm (Shimadzu SPD-6A UV spectrophotometric detector, Dyson Instruments Ltd., Tyne & Wear, UK). The chromatographs obtained were analysed using proprietary Shimadzu Class VP™ software.

2.7. Incubation with ALA and irradiation

Cells were harvested using 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA), resuspended in fresh medium and seeded in a 96-well plate with black walls and a clear bottom (Nalge Nunc International, Rochester, NY, USA). The initial cell concentration was 1.0×10^4 cells per well. Cells were then reincubated for 48 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- and M-ALA-containing media was adjusted to pH 7.4 and used immediately after preparation, due to known instability of ALA at this pH in cell culture media [17]. ALA and M-ALA were separately dissolved in were freshly prepared in LOX serum-free medium (SFM), a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 medium supplemented with 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, penicillin (1.0 IU ml^{-1}) and streptomycin (1.0 μg ml^{-1}). The pH of the solution was adjusted to pH 7.4 using 10 M sodium hydroxide solution. The stock solution was sterilised using appropriate filtration. Serial dilutions were performed under aseptic conditions, using LOX serum-free media, to give ALA and M-ALA solutions of concentrations; 10, 1.0, 0.1 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 LOX SFM. LOX SFM (100 μl), containing ALA, M-ALA or drug-free, was then added to each well. This medium is preferred because serum is known to cause efflux of PpIX from cultured cells [18]. 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- and M-ALA-containing dosage forms used in clinical application. A separate 96-well plate was used for each ALA or M-ALA concentration. On each plate, 12 wells contained ALA or M-ALA and were irradiated, 12 wells contained ALA or M-ALA and were not irradiated, 12 wells did not contain drug and were irradiated and 12 wells did not contain drug 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 LOX medium containing 10% v/v FCS and the plate returned to the incubator for 20 h.

2.8. 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 [19]. 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.

2.9. 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 Section 2.7, except that all 60 available wells had ALA- or M-ALA-containing SFM added.

Again, separate plates were used for each ALA or M-ALA concentration and a blank plate, with only SFM added, was used. Instead of irradiation being performed after 4 h of incubation, plates were removed from the incubator at regular, pre-defined, intervals. At each interval, the serum-free media were 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 monomerise porphyrins, so that their concentration can be reliably determined by fluorescence measurements [20]. 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 wavelength was set at 635 nm, using a long-pass cut-off filter (530 nm) to remove unwanted, scattered light. PpIX was quantified with respect to a standard curve (range 6–9000 ng ml^{-1}) and PpIX levels were expressed as ng μg^{-1} of protein, determined using the BCA assay described in Section 2.10.

2.10. Protein assay

Cells incubated, as described in Section 2.7, but not irradiated, were measured for protein content by the bicinchoninic acid (BCA) protein assay. LOX SFM 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 pelletise 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 125–1000 μg ml^{-1}).

2.11. Statistical analysis

For the drug release experiments, data were analysed, where appropriate, using a one-way Analysis of Variance (ANOVA). Post hoc comparisons were made using Fisher's PLSD test. The influences of different ALA or M-ALA concentrations on the number of surviving LOX cells were analysed using the Kruskal–Wallis test. This test was also used to analyse the influences of increasing ALA or M-ALA concentrations on the amount of protoporphyrin IX produced by cells. The Wilcoxon signed-rank test was used to compare the differing effects of ALA and M-ALA on the amount of protoporphyrin IX produced by cells and on the number of surviving LOX cells. This test was also used to analyse the effect of irradiation on the number of surviving LOX cells. In all cases, $p < 0.05$ denoted significance.

3. Results

The retention time of the fluorescent ALA derivative was 6 ± 0.53 min ($n = 5$). The derivative HPLC calibration curve for ALA was linear in the concentration range investigated, which was from 50 to 500 μg ml^{-1} of ALA in the original samples. The limit of detection was 0.05 μg ml^{-1}. Tables 1 and 2 show the validation details for the analytical methods used in the determination of both ALA and M-ALA. The retention time of the fluorescent M-ALA derivative was 8 ± 0.21 min ($n = 5$). The derivative HPLC calibration curve for M-ALA was linear in the concentration range investigated, which was from 0.6 to 600 μg ml^{-1} of M-ALA in the original samples. The limit of detection was 17.82 μg ml^{-1}. Samples taken during ALA release across Cuprophan® were diluted 1 in 20 prior to derivatisation. This was done so as not to exceed the reaction capacity of the derivatisation reagent and to allow placement on the calibration plot. All other samples were derivatised undiluted.

Table 3 shows the mean (±SD) weights of cream applied per square centimetre. Table 3 also shows the mean ALA and M-ALA doses available per square centimetre. An ALA loading of 38 mg cm^{-2} was, therefore, used as a starting point for patch design. ALA loadings of 19, 38 and 50 mg cm^{-2} were all investigated with respect to the

Table 1
Calibration curve properties for the analytical methods used in this study as determined by linear regression and correlation analyses of representative calibration plots produced from calibration experiments performed on three separate days

Slope	*y*-Intercept	r^2	RSS	LOD (μg ml^{-1})	LOQ (μg ml^{-1})
ALA					
32811	−31,429	0.9964	2.6×10^{12}	0.05	0.14
M-ALA					
7661.6	−14,803	0.9994	3.8×10^{10}	17.82	54.01

The limits of detection (LOD) and quantification (LOQ) of 5-aminolevulinic acid and 5-aminolevulinic acid methyl ester allowed for by this method were determined using the representative calibration plots as recommended by the International Conference on Harmonisation [59]. $LOD = 3.3\sigma/S$ and $LOQ = 310\sigma/S$, where σ is the standard deviation of the response (peak area) of the data used to construct the regression line and S is the slope of that line.

Table 2
Assay variability for fluorimetric determination of 5-aminolevulinic acid and its methyl ester following derivatisation

Selected concentration (μg ml^{-1})	Mean concentration found (μg ml^{-1})	SD	CV (%)	Accuracy (%) ($n = 5$)
Inter-day (ALA)				
200	181.77	0.81	0.44	90.89
500	515.03	5.44	1.06	103.01
Intra-day (ALA)				
200	181.7059	0.23	0.13	90.85
500	512.16	4.21	0.82	102.43
Inter-day (M-ALA)				
300	307.3461	0.519355	0.16898	102.4487
600	610.2551	6.045816	0.990703	101.7092
Intra-day (M-ALA)				
300	309.5456	2.464219	0.796076	103.1819
600	606.9003	5.1305	0.845361	101.15

The inter-day variation of the analysis was investigated by separately injecting samples of high and low ALA or M-ALA concentrations onto the column on five different days. The intra-day variation of the analysis was investigated by separately injecting samples of high and low ALA or M-ALA concentrations onto the column on five different occasions on the same day. The mean concentration determined (±SD), the coefficient of variation (CV) and the percentage accuracy of the method were reported in all cases.

Table 3
Outcome of experiment to determine approximate ALA and M-ALA loadings for a bioadhesive patch (mean ± SD, $n = 25$)

Mean mass of cream applied (g cm^{-2}) (±SD)	ALA delivered by the 20% w/w proprietary cream (approximate amount) (mg cm^{-2})	M-ALA delivered by the 16% w/w proprietary cream (approximate amount) (mg cm^{-2})
0.19 ± 0.05	38.00 ± 0.01	30.40 ± 0.01

influence of different ALA contents on drug release across model membranes and excised *stratum corneum*. In addition, an M-ALA loading of 30.4 mg cm^{-2} was used as a starting point for patch design. M-ALA loadings of 15.2, 30.4 and 40 mg cm^{-2} were all investigated with respect to the influence of different M-ALA contents on drug release across model membranes and excised *stratum corneum*.

Fig. 2 compares the ALA concentrations achieved in receiver compartments during release of the drug across Cuprophan® membranes from bioadhesive patches and the proprietary cream. Table 4 compares the different formulations in terms of maximum ALA concentrations achieved in the receiver compartments, total masses of drug released over 6 h and percentage of total drug released over the same time period. The proprietary Porphin® cream yielded a concentration of approximately 15.4 mM in receiver compartments after 6 h. This was significantly greater ($p = 0.0001$) than that produced by the patch containing 19 mg cm^{-2} ALA (10.4 mM). However, the amount of cream applied to the membrane in the donor compartment (0.34 g) contained approximately twice as much drug as the area of patch applied to the same area of membrane (1.77 cm^2). The patch containing 38 mg cm^{-2} ALA, which contains the same amount of drug in a 1.77 cm^2 area as 0.34 g of cream, yielded a significantly greater ($p < 0.0001$) receiver compartment concentration of approximately 21.1 mM after 6 h release. The patch containing 50 mg cm^{-2} ALA produced a receiver compartment concentration of approximately 27.6 mM, which was significantly greater than that produced by both the proprietary cream ($p < 0.0001$) and the 38 mg cm^{-2} patch ($p < 0.0001$). The cream only released approximately 42% of its total loading across Cuprophan® after 6 h. Each of the patches released approximately 57% of their individual total loadings in the same time period.

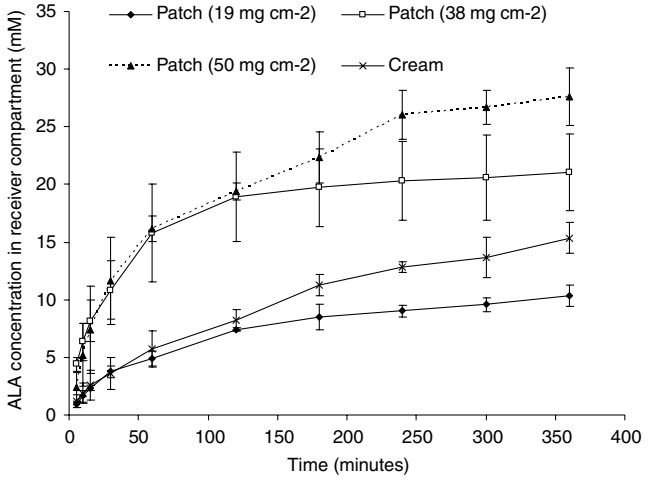

Fig. 2. Influence of formulation type, drug loading and time on ALA concentrations achieved in receiver compartments when patches and the proprietary Porphin® cream were applied to Cuprophan® membranes. Means (±SD), $n = 5$.

Fig. 3(A) compares the ALA concentrations achieved in receiver compartments during release of the drug across *stratum corneum* membranes from bioadhesive patches. It is clear that ALA exhibits typical membrane-controlled diffusion across excised *stratum corneum*, with receiver compartment concentrations increasing linearly with time. From Table 4, it can be seen that patches containing 19 and 38 mg cm^{-2}, respectively, did not significantly differ

Table 4
Influence of formulation type, drug loading and membrane type on maximum ALA concentration achieved in receiver compartment, mass of ALA released after 6 h and percentage of total ALA released after 6 h

Formulation	Membrane	Maximum concentration achieved in receiver compartment (mM)	Mass of ALA released after 6 h (μg)	% of total ALA released after 6 h
Porphin® cream	Cuprophan®	15.37 ± 1.36	28102.99 ± 1128.35	41.85 ± 1.68
Patch (19 mg cm^{-2})	Cuprophan®	10.36 ± 0.92	19064.01 ± 2793.24	57.78 ± 8.32
Patch (38 mg cm^{-2})	Cuprophan®	21.06 ± 3.36	38315.15 ± 2105.84	57.06 ± 3.14
Patch (50 mg cm^{-2})	Cuprophan®	27.60 ± 2.52	50430.23 ± 564.23	57.08 ± 0.64
Porphin® cream	*Stratum corneum*	0.37 ± 0.04	731.83 ± 80.50	1.09 ± 0.12
Patch (19 mg cm^{-2})	*Stratum corneum*	0.04	68.07 ± 5.79	0.20 ± 0.02
Patch (38 mg cm^{-2})	*Stratum corneum*	0.04	75.66 ± 6.81	0.11 ± 0.01
Patch (50 mg cm^{-2})	*Stratum corneum*	0.06	120.79 ± 12.08	0.14 ± 0.01

Means (±SD), $n = 5$.

($p = 0.1711$) in terms of the maximum concentrations achieved in receiver compartments after 6 h, which were approximately 0.04 mM. Patches containing 50 mg cm^{-2} ALA allowed concentrations of approximately 0.06 mM to be achieved in receiver compartments, which were significantly greater than those achieved by both the patches containing 19 mg cm^{-2} ($p < 0.0001$) and 38 mg cm^{-2} ($p < 0.0001$).

Fig. 3(B) compares the release of ALA from the patches with that from the proprietary cream across *stratum corneum*. It is clear that the cream released significantly more drug across *stratum corneum* than any of the patches over 6 h. An initial linear phase of diffusion of ALA from the cream across *stratum corneum* was observed, followed by an exponential release phase from 180 to 360 min. As can be seen from Table 4, the cream allowed an ALA concentration of approximately 0.4 mM to be achieved in receiver compartments. This was significantly greater than that achieved by any of the patches. The proprietary cream released approximately 1.1% of its total loading across *stratum corneum* over 6 h. The patches released only between 0.1% and 0.2% of their total loadings across *stratum corneum* over the same period. In all cases, the concentrations achieved in the receiver compartments and percentages of total drug released were significantly less when *stratum corneum* was used as the barrier membrane, rather than Cuprophan®.

The linear diffusion of ALA across *stratum corneum* enabled calculation of flux of the drug through, and diffusivity in, the barrier. The flux (J), an expression of the mass of ALA (dM) flowing through a *stratum corneum* barrier membrane of unit cross-sectional area (S), in unit time (dt), was calculated from the data obtained using Fick's First Law

$$J = \frac{dM}{S dt}. \quad (1)$$

The diffusivity (D) of the drug in *stratum corneum* was calculated using

$$J = -D\frac{dC}{dx} \quad (2)$$

knowing the thickness of the *stratum corneum* (dX) and the calculated flux. The concentration gradient (dC) was calculated using the experimental data obtained and knowledge of the original drug concentration in the patch. Dried patches were approximately 1 mm thick and, since, for example, they contained 50 mg cm^{-2} ALA, the original drug concentration was 500 mg cm^{-3}.

As can be seen from Table 5, Porphin® cream generated an ALA flux of approximately 4.5×10^{-4} mg cm^{-2} s^{-1} across *stratum corneum*. This was significantly greater than the fluxes generated by the patches, which were in the range $1.0–2.0 \times 10^{-4}$ mg cm^{-2} s^{-1}. The ALA diffusivity in *stratum corneum* generated by the cream, at approximately 1.3×10^{-7} cm^2 s^{-1}, was, again, significantly greater than those generated by the patches, which were in the range $2.0–3.0 \times 10^{-8}$ cm^2 s^{-1}.

Fig. 4 compares the M-ALA concentrations achieved in receiver compartments during release of the drug across Cuprophan® membranes from bioadhesive patches and the proprietary cream. Table 6 compares the different formulations in terms of maximum M-ALA concentrations achieved in the receiver compartments, total masses of drug released over 6 h and percentage of total drug released over the same time period. The proprietary Metvix® cream yielded a concentration of approximately 2.1 mM in receiver compartments after 6 h. This was significantly greater ($p = 0.0186$) than that produced by the patch containing 15.2 mg cm^{-2} M-ALA (1.0 mM). However, the amount of cream applied to the membrane in the donor compartment (0.34 g) contained approximately twice as much drug as the area of patch applied to the same area of membrane (1.77 cm^2). The patch containing 30.4 mg cm^{-2} M-ALA, which contains the same amount of drug in a 1.77 cm^2 area as 0.34 g of cream, yielded a significantly greater ($p = 0.0469$) receiver compartment concentration of approximately 3.0 mM after 6 h release. The patch containing 40 mg cm^{-2} M-ALA produced a receiver compartment concentration of approximately 5.2 mM, which was significantly greater than that produced by both the proprietary cream ($p < 0.0001$) and the 30.4 mg cm^{-2} patch ($p = 0.0001$). The cream only released approximately 6.5% of its total loading across Cuprophan® after 6 h,

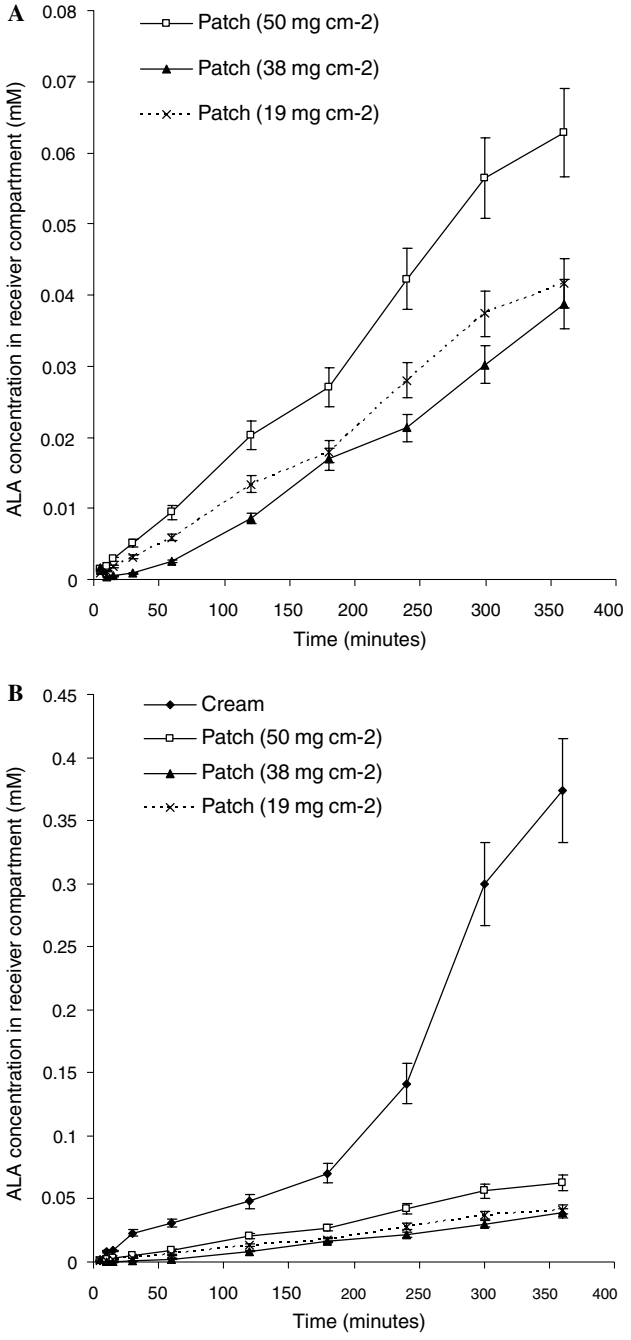

Fig. 3. Influence of drug loading and time on ALA concentrations achieved in receiver compartments when patches were applied to *stratum corneum* membranes (A). Comparison of ALA concentrations achieved in receiver compartments after release across *stratum corneum* membranes from patches and the proprietary Porphin® cream over time (B). Means (±SD), $n = 5$.

while patches containing 15.2 and 30.4 mg cm^{-2} M-ALA released approximately 6.7% and 5.6% of their total loadings, respectively. In contrast, the patch containing 40 mg cm^{-2} M-ALA released approximately 13.5% of its total loadings in the same time period. In all cases, the concentrations achieved in the receiver compartments and percentages of total drug released were significantly less for M-ALA than for ALA.

Fig. 5(A) compares the M-ALA concentrations achieved in receiver compartments during release of the drug across *stratum corneum* membranes from bioadhesive patches. In contrast to ALA, it is clear that M-ALA does not exhibit typical membrane-controlled diffusion across excised *stratum corneum*. From Table 6, it can be seen that patches containing 15.2 mg cm^{-2} M-ALA allowed concentrations of approximately 0.02 mM to be achieved in receiver compartments after 6 h. These were not significantly different to the approximate concentrations of 0.03 mM achieved by both the patches containing 40 mg cm^{-2} ($p = 0.52228$) and 30.4 mg cm^{-2} ($p = 0.5036$).

Fig. 5(B) compares the release of M-ALA from the patches with that from the proprietary cream across *stratum corneum*. It is clear that the cream released significantly more drug across *stratum corneum* than any of the patches over 6 h. An initial lag phase of diffusion of M-ALA from the cream across *stratum corneum* was observed, followed by an exponential release phase from 120 to 360 min. As can be seen from Table 6, the cream allowed an M-ALA concentration of approximately 0.83 mM to be achieved in receiver compartments. This was significantly greater than that achieved by any of the patches. The proprietary cream released approximately 2.5% of its total loading across *stratum corneum* over 6 h. The patches released only approximately 0.1% of their total loadings across *stratum corneum* over the same period. In all cases, the concentrations achieved in the receiver compartments and percentages of total drug released were significantly less when *stratum corneum* was used as the barrier membrane, rather than Cuprophan®. Metvix® released significantly more M-ALA across *stratum corneum* than Porphin® did ALA over 6 h ($p < 0.0001$). Each ALA patch released more drug across *stratum corneum* than the corresponding M-ALA patch. The difference was, however, only significant for the 50 mg cm^{-2} ALA patch, which released significantly more ($p = 0.0401$) drug across *stratum corneum* than the corresponding 40 mg cm^{-2} M-ALA patch.

Due to the absence of a linear portion in any of the mass of drug released across *stratum corneum* versus time plots, flux of M-ALA through and diffusivity of the drug in, *stratum corneum* could not be accurately determined.

Fig. 6 shows the influences of increasing concentrations of ALA and M-ALA and irradiation on the survival rates in wells containing approximately 1.0×10^4 viable LOX cells. The numbers of viable cells, as determined using the MTT assay, were shown to remain significantly unchanged ($p = 0.9672$) as the ALA concentration was increased to 100 mM and irradiated was not used. However, incubating cells with increasing concentrations of M-ALA caused a significant ($p = 0.0121$) reduction in the number of viable cells, such that only 63% of the original viable cells incubated with 100 mM concentrations remained. Exposure to light alone was shown to be an innocuous process, as demonstrated by no significant reductions ($p = 0.108$) in the viable cell count, when compared to control incubations free of ALA, M-ALA and light radiation. Incubation of

Table 5
Influence of formulation type and drug loading on ALA flux through, and diffusivity in, *stratum corneum* membranes

Formulation	Time period for linear region (min)	Mean R^2 value for linear region	ALA flux (mg cm^{-2} s^{-1})	ALA diffusivity (cm^2 s^{-1})
Porphin® cream	0–180	0.9478	$4.54 \pm 0.50 \times 10^{-4}$	$1.26 \pm 0.14 \times 10^{-7}$
Patch (19 mg cm^{-2})	0–360	0.9923	$1.07 \pm 0.09 \times 10^{-4}$	$2.96 \pm 0.25 \times 10^{-8}$
Patch (38 mg cm^{-2})	0–360	0.9738	$1.09 \pm 0.10 \times 10^{-4}$	$1.94 \pm 0.17 \times 10^{-8}$
Patch (50 mg cm^{-2})	0–360	0.9925	$1.91 \pm 0.19 \times 10^{-4}$	$2.20 \pm 0.22 \times 10^{-8}$

Means (±SD), $n = 5$.

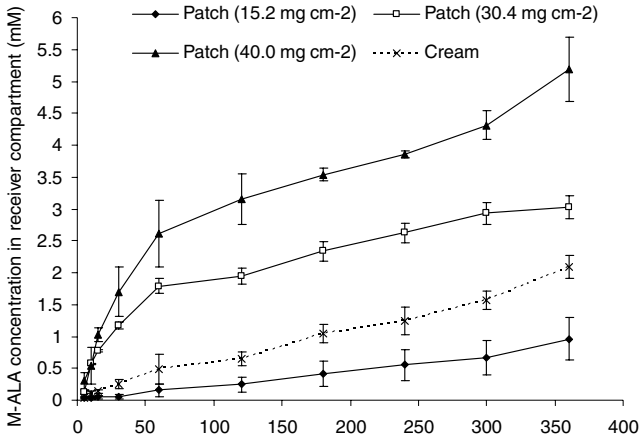

Fig. 4. Influence of formulation type, drug loading and time on M-ALA concentrations achieved in receiver compartments when patches and the proprietary Metvix® cream were applied to Cuprophan® membranes. Means (±SD), $n = 5$.

cells with 0.01 mM ($p = 0.2850$) or 0.1 mM ($p = 0.1088$) ALA for 4 h, followed by irradiation, caused no significant decrease in the number of viable cells. However, incubation with higher concentrations caused a progressive significant reduction in viable cell numbers. Indeed, increasing the ALA concentration to 100 mM caused a profound decrease in the number of viable cells following irradiation, such that only around 14% of the original viable population remained ($p = 0.0277$). Irradiation significantly reduced the number of surviving cells following incubation with 1.0 ($p = 0.0495$), 10 mM ($p = 0.0495$) and 100 mM M-ALA ($p = 0.0495$). At concentrations of 100 mM ($p = 0.8273$), 10 mM ($p > 0.9999$) and 1.0 mM ($p > 0.9999$), ALA did not cause a significantly increased cell kill with respect to 100, 10 and 1.0 mM M-ALA following irradiation.

Fig. 7 shows the effect of increasing concentrations of ALA and M-ALA 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. Increasing ALA concentration from 0.0 to 0.1 mM ($p = 0.0679$) had no significant effect on the amount of PpIX formed. However, increasing the ALA concentration from 0.0 to 1.0 mM caused a significant increase ($p = 0.0117$) in the amount of PpIX formed after 4 h incubation. Further increasing the ALA concentration caused further significant increases in the amount of PpIX formed ($p = 0.0117$). The amount of PpIX formed after 4 h incubation in 1.0 mM ALA-containing media was approximately 700 ng mg^{-1} protein, while the amount formed after 4 h incubation in 100 mM ALA was approximately 1600 ng mg^{-1} protein. Apart from cells incubated at concentrations of 100 mM ($p = 0.0117$), M-ALA produced no significant difference in the amounts of PpIX formed at any particular concentration by ALA.

Fig. 8 shows the time related accumulative PpIX concentration in LOX cells when periods of time are considered beyond the clinically relevant 4 h application interval. The amounts of PpIX in cells incubated with 0.1 mM ALA or M-ALA did not change significantly over 24 h. Cells incubated with higher concentrations of each of the drugs showed significant increases in PpIX content up

Table 6
Influence of formulation type, drug loading and membrane type on maximum M-ALA concentration achieved in receiver compartment, mass of M-ALA released after 6 h and percentage of total M-ALA released after 6 h

Formulation	Membrane	Maximum concentration achieved in receiver compartment (mM)	Mass of M-ALA released after 6 h (μg)	% of total M-ALA released after 6 h
Metvix® cream	Cuprophan®	2.09 ± 0.18	3444.81 ± 422.59	6.41 ± 0.79
Patch (15.2 mg cm^{-2})	Cuprophan®	0.96 ± 0.33	1800.32 ± 746.56	6.70 ± 2.80
Patch (30.4 mg cm^{-2})	Cuprophan®	3.02 ± 0.18	4940.20 ± 298.55	5.56 ± 0.56
Patch (40.0 mg cm^{-2})	Cuprophan®	5.19 ± 0.51	9566.34 ± 1616.03	13.54 ± 2.59
Metvix® cream	*Stratum corneum*	0.83 ± 0.09	1355.34 ± 149.01	2.52 ± 0.53
Patch (15.2 mg cm^{-2})	*Stratum corneum*	0.02	30.34 ± 2.46	0.11 ± 0.01
Patch (30.4 mg cm^{-2})	*Stratum corneum*	0.03	48.16 ± 4.33	0.09 ± 0.01
Patch (40.0 mg cm^{-2})	*Stratum corneum*	0.03	55.45 ± 5.55	0.08 ± 0.01

Means (±SD), $n = 5$.

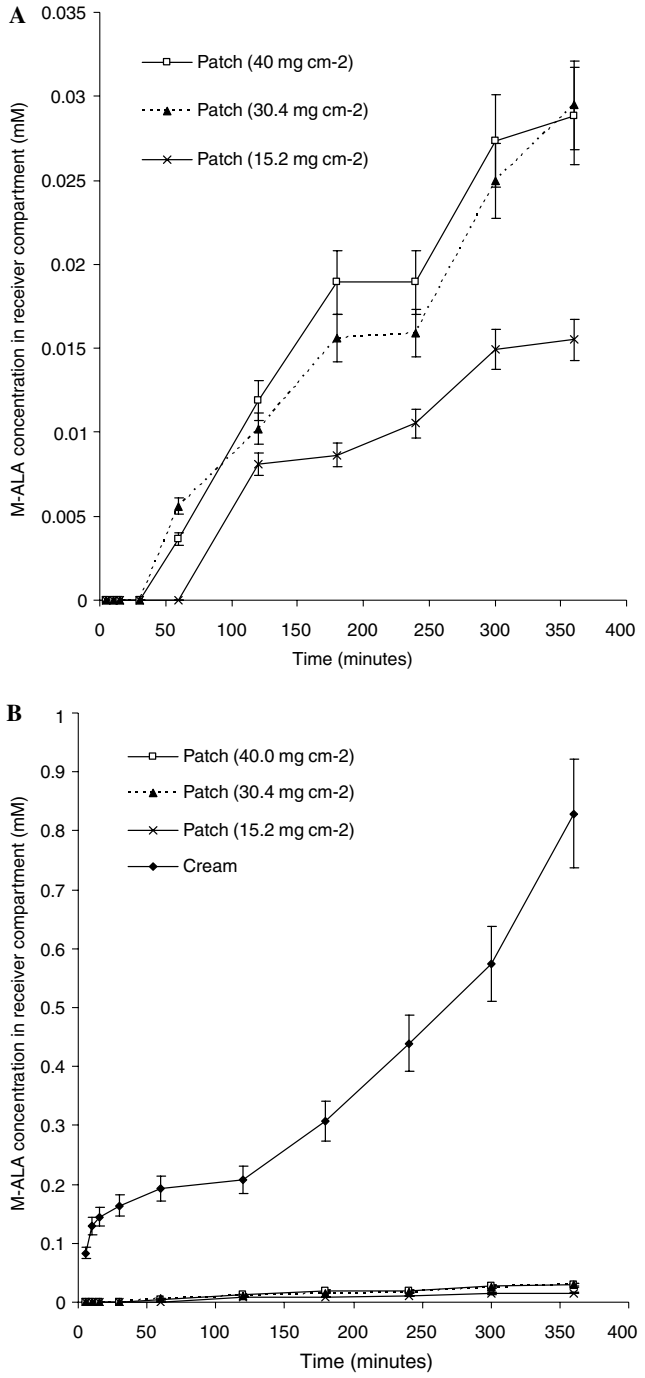

Fig. 5. Influence of drug loading and time on M-ALA concentrations achieved in receiver compartments when patches were applied to *stratum corneum* membranes (A). Comparison of M-ALA concentrations achieved in receiver compartments after release across *stratum corneum* membranes from patches and the proprietary Metvix® cream over time (B). Means (±SD), $n = 5$.

to 16 h, when production reached a plateau. There were no significant differences in amounts of PpIX produced by cells incubated with either of the two drugs at any time point, except at concentrations of 100 mM. Incubation with 100 mM ALA for 24 h produced approximately 3400 ng PpIX mg^{-1} protein in LOX cells, which was significantly greater than the 3200 ng PpIX mg^{-1} protein

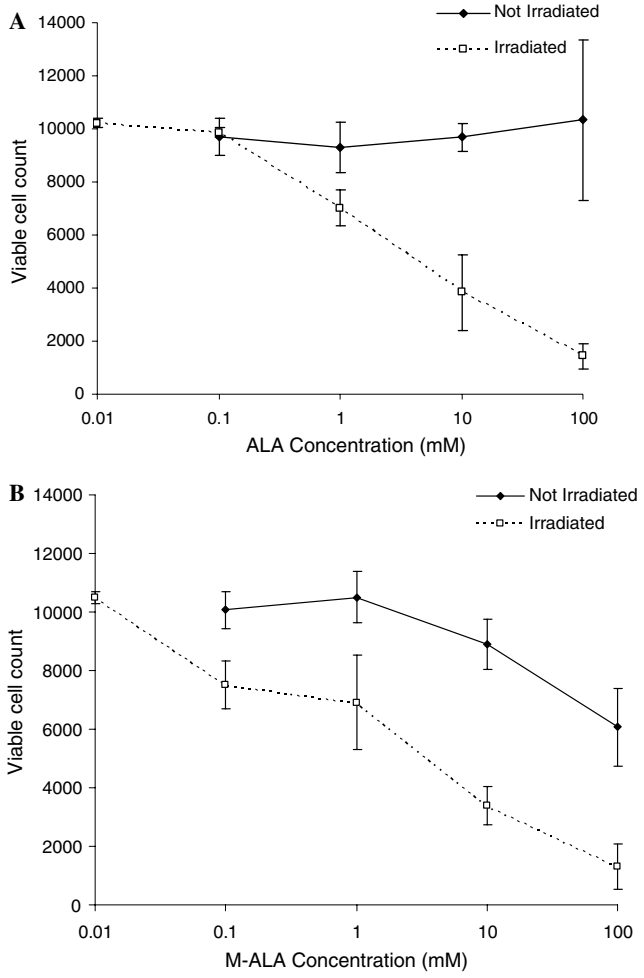

Fig. 6. Influences of increasing ALA (A) and M-ALA (B) concentrations and irradiation with 100 J cm^{-2} of red light (635 nm) on the numbers of surviving LOX cells in wells in 96-well plates originally containing approximately 10,000 viable cells. Cells were incubated with ALA or M-ALA for 4 h before irradiation. Means (±SD), $n = 12$.

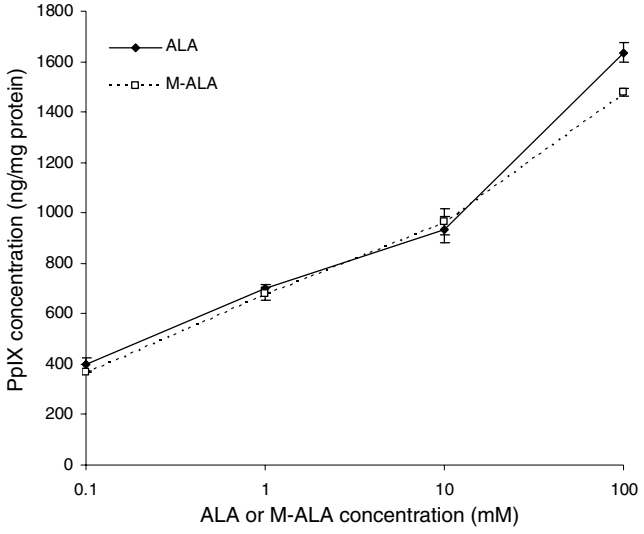

Fig. 7. The concentration dependence of protoporphyrin IX production in LOX cells after exposure to increasing amounts of ALA or M-ALA. The incubation time used was 4 h. Means (±SD), $n = 5$.

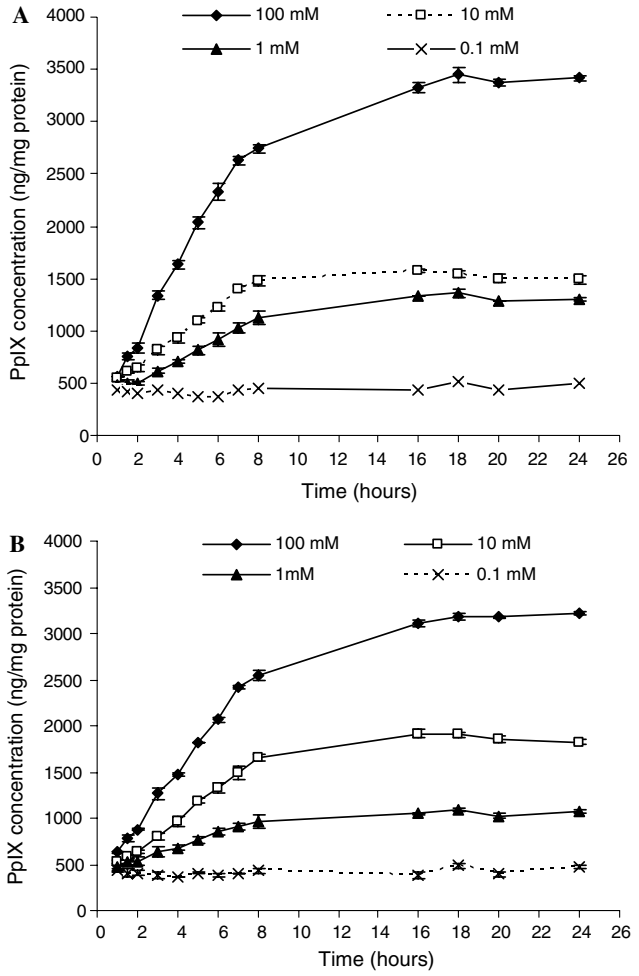

Fig. 8. PpIX production in LOX cells over a 24 h period in the presence of increasing concentrations of ALA (A) or M-ALA (B). Means (±SD), $n = 5$.

($p = 0.0277$) produced by incubation with 100 mM M-ALA for 24 h.

4. Discussion

Conventional treatments for skin neoplasias include surgical excision and radiotherapy, which are highly successful, with clearance rates as high as 95% on 5 years follow-up reported. However, these treatments are unsuitable for large or multiple lesions and can lead to poor cosmesis [1]. Cryotherapy and curettage are only suitable for very superficial tumours and are associated with high rates of recurrence. The most commonly used drug for the treatment of skin neoplasias by PDT is topically applied ALA, combined with red light.

Superficial skin lesions show an excellent response to topical ALA PDT but the response of deeper or hyper keratotic lesions is, in the main, disappointing. Such findings may reflect the poor penetration of ALA through keratin or even the absorption of the drug by keratotic debris overlying lesions [7,21,22].

Potential strategies for successful PDT of deeper lesions include multiple treatments or prolonged ALA application times [23,24]. Alternative strategies include employing penetration enhancers or curettage to allow ALA to penetrate deeper into the dermis within reasonable application times. Another option would be to use ALA esters, which are more lipophilic and should penetrate to greater depths in tissue than their more hydrophilic parent.

The most commonly used topical delivery systems for ALA and M-ALA are the proprietary o/w creams Porphin® (20% w/w ALA) and Metvix® (16% w/w M-ALA), respectively. There is currently little consensus as to the optimum amount of cream required for successful treatment. In fact, the dose of cream applied in clinical studies is rarely reported. Where the dose is detailed, great variability between studies is observed. Literature reports have indicated that between 10 and 200 mg of the ALA- or M-ALA-containing vehicle is applied per square centimetre of lesion, a 20-fold difference in drug dose [14,15,2,6,7,10,16,17]. The use of occlusive dressings to aid retention and enhance absorption can further increase this variability by causing smudging and subsequent spreading of the cream from the site of application. As a result, comparing the outcomes of different studies becomes very difficult. The use of a unit-dose system for topical delivery of ALA or M-ALA would eliminate this inter-clinician variability, enabling comparison of different studies. Moreover, if such a system were self-adhesive and backed with an occlusive material, the need for retentive dressings would be eliminated. This would be of particular benefit in the PDT of oral lesions or those of the lower female reproductive tract, where use of creams and occlusive dressings is impractical.

The mean amount of cream applied per cm^2 found in the present study, at 190 mg, falls within the upper reaches of the range detailed above. Knowing the drug loadings in the respective creams, this allowed calculation of the required amount of drug in the patches. A patch containing 38 mg cm^{-2} ALA, or 30.4 mg cm^{-2} M-ALA, should contain the same available dose as the respective cream. Drug loadings either side of these were also investigated to reflect the variability in clinically applied does of cream.

Cuprophan® is a dialysis membrane, with a molecular weight cut-off of 10,500 Da [25]. In simple terms, this means that it consists of a lipophilic polymeric membrane, interspersed with small pores filled by the aqueous receiver phase. It may, to some extent, approximate the disordered *stratum corneum* overlying neoplastic skin lesion. Small water-soluble molecules, such as ALA, should be able to pass freely through such a barrier. Patches containing ALA all released approximately 57% of their drug loadings across Cuprophan® membranes over 6 h, as may be seen from Fig. 2 and Table 4. The receiver compartment concentrations of ALA achieved varied from 10.4 mM, for the 19 mg cm^{-2} patch, to 27.6 mM, for the 50 mg cm^{-2} patch over the same period. Porphin® released only approximately 42% of its drug loading after 6 h and the

maximum concentration achieved in the receiver compartment was 15.4 mM. The reason for the reduced release from the cream may be due to the chemical nature of the drug and vehicle. ALA is water soluble, with an octanol:water partition coefficient of 0.03 [27]. The Unguentum Merck® base of Porphin® has a high lipid content [26]. Consequently, diffusion of ALA through the cream base is likely to be much slower than that through the hydrophilic matrix of the patch.

In all cases, the corresponding M-ALA formulation released significantly less drug than its ALA-containing equivalent. This may be due, in part, to the slightly lower drug loadings in the M-ALA systems, which were based on the 16% w/w Metvix® cream, as compared to the 20% w/w Porphin® cream. The most likely explanation, however, is due to the enhanced octanol:water partition coefficient of M-ALA (0.12) compared to that of ALA [27]. The more lipophilic drug is more likely than its hydrophilic parent to partition into the lipophilic polymer of the barrier membrane. The majority of the M-ALA released is still likely to move through the aqueous-filled pores. However, the partitioning of a significant amount of drug into the membrane may slow its diffusion into the receiver compartment and, thus, explain the lower concentrations and percentages of total drug released observed. Metvix® released significantly less M-ALA across Cuprophan® than the corresponding ($30.4\,mg\,cm^{-2}$) patch over 6 h. Since lipophilicity is unlikely to be the reason here, this may be due to the fact that the 336 mg of cream applied to the $1.77\,cm^2$ membrane occupies a significant volume. Once drug at the release surface is depleted, M-ALA from further up in the cream must diffuse to the surface for release. In contrast, formed patches were approximately only 1 mm thick, thus limiting the diffusional distance for drug not at the surface.

With respect to the physiochemical properties of a drug, it is generally accepted that lipophilicity and molecular weight of the compound are the primary determinants of its ability to penetrate the *stratum corneum* [28]. The *stratum corneum*, due to the lipid character of the intercellular route for drug diffusion, is not an accessible phase for either very polar or charged species. Chemical derivatisation of ALA was pioneered by the Moan group in Oslo in the mid-1990s [29]. Since then, numerous ALA pro-drugs have been synthesised, employing reaction at the amino [30,31] or, most commonly, the carboxylic acid group [32,33] of the parent ALA molecule. The aim, in each case, was to produce a pro-drug with greater lipophilicity than ALA and, in theory, greater tissue penetration and efficacy in PDT.

The literature contains many reports on the clinical use of ALA and M-ALA and also on in vitro cell culture investigations of the compounds. However, very few publications concerning the penetration of these agents across *stratum corneum* exist. In the present study, the concentrations of ALA and M-ALA achieved on the receiver compartment side of *stratum corneum* were significantly less than those achieved when Cuprophan® was the barrier membrane, regardless of the formulation. Differences as high as two orders of magnitude were observed in the ALA study, and with the M-ALA patches, as may be noted from Tables 2 and 4. The penetration profile of ALA across *stratum corneum* was typical of membrane-controlled diffusion [34]. The linear relationship between mass of drug released and time allowed calculation of ALA flux through, and diffusivity in, *stratum corneum*. The flux of ALA released from the cream across *stratum corneum* was approximately $4.5 \times 10^{-4}\,mg\,cm^{-2}\,s^{-1}$, while that from the patches was between 1.0 and $2.0 \times 10^{-4}\,mg\,cm^{-2}\,s^{-1}$. These values are significantly greater than those reported elsewhere. Winkler et al. [35] reported an ALA flux across *stratum corneum* of approximately $2.9 \times 10^{-8}\,mg\,cm^{-2}\,s^{-1}$. However, these authors used a lower ALA concentration (10% w/w) and, perhaps more importantly, used a w/o cream for delivery of the drug. The diffusion of the hydrophilic ALA through such a hydrophobic cream matrix is likely to be poor. De Rosa et al. [33] and Tsai et al. [36] report the flux of ALA through full thickness mouse skin to be approximately 1.2×10^{-9} and $1.9 \times 10^{-9}\,mg\,cm^{-2}\,s^{-1}$, respectively. The low values obtained are likely to be due to the significantly thicker barrier for drug diffusion to that studied here. The release of M-ALA across *stratum corneum* yielded a diffusion pattern more closely resembling a square root of time release than membrane-controlled diffusion. This may suggest formation of an M-ALA reservoir in the *stratum corneum*, with subsequent slow diffusion into the receiver compartment, as suggested by Lopez et al. [37].

Each of the creams released significantly more drug across *stratum corneum* than the corresponding patches. Indeed, the calculated diffusivity of ALA through *stratum corneum* was an order of magnitude greater for the cream than for the patches. Both creams possess high lipid contents, despite their o/w natures [26,38]. Compounds, such as cetostearyl alcohol, glyceryl stearate and caprylic triglyceride contained in Porphin®, and oleyl alcohol and cholesterol contained in Metvix®, may act as penetration enhancers, solubilising and disrupting intercellular lipids and, thus enhancing drug penetration [39]. No such compounds were present in the patch formulations. In fact, due to its hydrophilicity, ALA may preferentially remain in the water-based patch when presented with a lipophilic barrier, such as the *stratum corneum*.

Metvix® released significantly more M-ALA across *stratum corneum* than Porphin® did ALA over 6 h. This may be due to the greater lipophilicity of M-ALA, which has a *stratum corneum*:water partition coefficient of 1.6, compared to that of ALA, which is 0.04 [27]. However, and interestingly, each ALA patch released more drug across *stratum corneum* than the corresponding M-ALA patch. The difference was, however, only significant for the $50\,mg\,cm^{-2}$ ALA patch, which released significantly more drug across *stratum corneum* than the corresponding

40 mg cm^{-2} M-ALA patch. These slight differences are possibly due to the slightly greater ALA loadings.

It is possible that there is no significant difference in *stratum corneum* permeability of the two drugs when delivered using the patch system described here. Reservoir formation in the *stratum corneum* by M-ALA may limit the amount of drug entering the receiver compartment over short time periods, such as those studied here. The differences in drug penetration observed between the creams may simply be due to the differing abilities of the cream bases to enhance drug penetration. Indeed, it has been shown that within clinically relevant application times, such as 4 or 6 h, no significant difference is observed in amounts of ALA or ALA-esters penetrating *stratum corneum*, regardless of ester alkyl chain length [33,35,40]. Several in vivo studies have investigated PpIX production in the skin of human volunteers [41–43] or nude mice [29,44–46] following topical application of ALA or one of its esters. Again, significant lag times are generally observed before PpIX fluorescence induced by ALA esters becomes greater than that induced by ALA.

The model membranes used in the present study present two extremes in terms of diffusion of ALA and M-ALA across the disordered *stratum corneum* overlying neoplastic lesions. Cuprophan® is likely to be much more permeable to drugs released from topical formulations than is disordered *stratum corneum*. In contrast, the normal *stratum corneum* barrier employed here is likely to be much more impermeable to released drugs. The true picture is likely to be in between the two extremes and can only be obtained by using actual neoplastic lesions excised from patients. Indeed, we have previously shown that ALA concentrations as high as 70 mM can be induced at depths of 2.0 mm in nodular BCCs after a 4 h application of Porphin® cream [47].

If topically applied ALA and M-ALA are to be successfully used for PDT, their concentrations, and, hence, that of the photosensitising protoporphyrin IX (PpIX) which they generate, in viable neoplastic cells below the *stratum corneum*, must exceed a threshold. If the threshold ALA or M-ALA concentration, which varies with the type of abnormal cell, is not exceeded then sufficient amounts of PpIX will not be produced. Irradiation of the target cells will then not generate enough singlet oxygen to successfully eradicate the lesion being treated. Cell culture experiments in vitro have demonstrated that concentrations of ALA must reach levels between 0.06 and 1.0 mM before sufficient protoporphyrin IX (PpIX) is produced to cause a satisfactory level of neoplastic cell death on illumination [19,27,48].

LOX was used as a neoplastic model cell line of dermatological origin in this work. Ideally, a primary culture from an actual BCC, Bowen's disease lesion or actinic keratosis would improve the experimental design. However, the lack of available transformed 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 LOX line makes it a reproducible working model. The pH of the culture media was not controlled for the purposes of maintaining ALA/M-ALA stability throughout the duration of the experiment. This approach has been adopted by other workers [19], and employed in this work primarily in an attempt to mimic in vivo conditions, where ALA/M-ALA is released into local tissue conditions and where pH is expected to be close to that of physiological. Arguably, increased ALA/M-ALA degradation is inevitable, but cellular quiescence, occurring at low pH values where ALA and M-ALA are stable [49,50], may make the interpretation of PpIX levels difficult.

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 LOX cells was strongly dependent on the ALA/M-ALA concentration. Cells incubated in media containing ALA/M-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 in 1.0 mM ALA or M-ALA produced approximately 700 ng PpIX mg^{-1} protein. This is of the same order as the amount of the photosensitiser formed in WiDr (recto-sigmoid colon adenocarcinoma) cells [48,51] and in bladder cancer cells [52], incubated for 4 h in 1.0 mM ALA. Only at concentrations of 100 mM did ALA induce significantly more PpIX in LOX cells than did M-ALA. This may be due to the toxic effect of M-ALA at this concentration reducing the number of cells producing the photosensitiser.

The effect of light by itself did not significantly alter the cell viability, as shown in Fig. 6. Upon irradiation and exposure to media containing ALA or M-ALA, reductions in the numbers of viable cells were observed. However, even concentrations of 100 mM could not reduce the viable cell count to levels lower than around 14%. In contrast, we have previously shown that ALA concentrations in excess of 1.0 mM were needed to reduce viable HeLa cell populations to 10% [53]. In addition, a reduction of 90% in the number of viable WiDr cells was achieved after 4 h incubation with 0.05 mM ALA and irradiation with a light dose of 3.6 J cm^{-2} at 635 nm [48]. 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 around 10 mM [53]. However, above this concentration, the drug itself became toxic to cells without the intervention of irradiation. In this study, even an ALA concentration of 100 mM caused no toxic effect to be observed in the absence of irradiation. M-ALA alone was toxic to LOX cells at concentrations above 1.0 mM. Irradiation did, however, significantly increase the kill rate, as can be seen from Fig. 6(B).

It is now clear that, in comparison to other model cell lines, LOX cells are quite resistant to ALA- and

M-ALA-induced phototoxicity. Consequently, neither cream nor patch formulations released sufficient ALA or M-ALA across Cuprophan® or *stratum corneum* membranes to induce the most phototoxic concentration of 100 mM in receiver compartments. However, the receiver compartment concentrations of approximately 28 mM achieved after 6 h release across Cuprophan® by the 50 mg cm^{-2} ALA patch and of approximately 1.0 mM achieved after 6 h release across *stratum corneum* by Metvix® are likely to induce significant phototoxic reactions in the other three model cell lines mentioned above [48,53,54]. Indeed, we have already described the successful clinical use of the 38 mg cm^{-2} ALA patch in the treatment of lesions of vulval intraepithelial neoplasia vulval Paget's disease and vulval squamous cell carcinoma [55–57].

The fact that the aqueous-based patches release significantly *less* drug across normal *stratum corneum* and significantly *more* across a model membrane designed to mimic the abnormal *stratum corneum* overlying neoplastic skin lesions is of major significance. This means that the selectivity of PDT for neoplastic lesions will be further enhanced by the delivery system, since ALA/M-ALA will only be delivered in significant amounts to the *abnormal* tissue. PpIX will only then accumulate in the neoplastic cells and the normal surrounding tissue will be unharmed upon irradiation. Indeed, we have recently observed [58] that the ALA-containing patch induces the same level of PpIX production in skin overlying subcutaneously implanted murine tumours, which are known to possess disordered *stratum corneum* barriers, as the cream but much *lower* levels in normal skin.

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