JPP Journal of Pharmacy and Pharmacology

JPP 2010, 62: 685–695

Received August 28, 2009
Accepted December 16, 2009
DOI 10.1211/jpp/62.06.0004
ISSN 0022-3573

Research Paper

Influence of penetration enhancers on topical delivery of 5-aminolevulinic acid from bioadhesive patches

Desmond I.J. Morrow[a,c], Paul A. McCarron[b], A. David Woolfson[a], Petras Juzenas[d], Asta Juzeniene[c], Vladimir Iani[c], Johan Moan[c] and Ryan F. Donnelly[a,c]

[a]School of Pharmacy, Queen's University Belfast, Medical Biology Centre, 97 Lisburn Road, Belfast, [b]Department of Pharmacy and Pharmaceutical Sciences, University of Ulster, Cromore Road, Coleraine, UK, [c]Department of Radiation Biology, Institute for Cancer Research, Rikshospitalet-Radiumhospitalet Medical Center, Montebello and [d]Institute of Physics, University of Oslo, Blindern 0316 Oslo, Norway

Abstract

Objectives The inclusion of chemical penetration enhancers in a novel patch-based system for the delivery of 5-aminolevulinic acid (ALA) was examined *in vitro* and *in vivo*. Poor penetration of ALA has been implicated as the primary factor for low response rates achieved with topical ALA-based photodynamic therapy of thicker neoplastic lesions, such as nodular basal cell carcinomas.

Methods Several chemical permeation enhancers (dimethylsulfoxide, Labrafac CC, Labrafac PG and Labrafil M1944CS) were incorporated into bioadhesive patches tailored to deliver 19 mg ALA/cm^2.

Key findings In-vitro depth penetration studies into excised porcine skin showed that high concentrations of ALA (>9 μmol/cm^3) could be delivered to a depth of 1.875 mm. However, inclusion of permeation enhancers did not significantly increase ALA delivery, relative to the control ($P > 0.05$). In-vivo studies were in strong agreement with in-vitro results, with formulations containing chemical enhancers showing no improvement in delivery compared with the control.

Conclusions The patches designed in this work are suited to defineable ALA delivery without the need to immobilise patients for up to 6 h, as is common with the cream-under-occlusion approach. Overall, permeation enhancers were not found to markedly enhance the topical delivery of ALA. However, chemical penetration enhancers may have a greater effect on the delivery of more lipophilic ALA prodrugs, which are thought to primarily permeate the stratum corneum via the intercellular pathway.

Keywords 5-aminolevulinic acid; bioadhesive patch; permeation enhancers; topical drug delivery

Introduction

Photodynamic therapy uses a combination of photosensitising drug and visible light to cause the selective destruction of neoplastic cells, primarily through the production of singlet oxygen.[1] Pre-formed photosensitisers generally lack the desirable physicochemical properties required to successfully permeate the skin barrier.[2] Alternatively, aminolevulinic acid (ALA), a small molecular weight (167.6 Da) precursor of the endogenous photosensitiser protoporphyrin IX (PpIX) can be topically applied. However, while the molecular weight of ALA is unlikely to affect its ability to penetrate skin, its zwitterionic nature will impair penetration, since it is ionised at both high and low pH. Furthermore, it has an octanol : water partition coefficient (log P_{ow}) of –1.5.[3] As a result, topically applied ALA penetrates intact stratum corneum poorly.[4,5] Although disordered epithelial barriers presented by many skin lesions allow enhanced ALA penetration, its low lipophilicity prevents it from penetrating significantly into hyperkeratotic or deep nodular lesions.[6]

Altering the composition and structure of the stratum corneum has been shown to significantly influence drug penetration into skin. One of the most common approaches of modifying this horny layer is to employ formulation-based chemical permeation enhancers.

Correspondence: Ryan F. Donnelly, School of Pharmacy, Queens University Belfast, Medical Biology Centre, 97 Lisburn Road, Belfast, BT9 7BL, UK.
E-mail: r.donnelly@qub.ac.uk

Permeation enhancers reduce the barrier resistance of the skin in a reversible and innocuous manner.[7] The lipid protein partitioning theory attributes the potential modes of action of penetration enhancers to one or more of three main mechanisms: (i) disruption of the highly ordered structure of stratum corneum lipids; (ii) interaction with intracellular protein; and (iii) improved partitioning of a drug, coenhancer or cosolvent into the stratum corneum.[8]

To date, several chemical permeation enhancers have been investigated for enhancing topical delivery of ALA including, dimethylsulfoxide (DMSO),[5] 1-[2-(decylthio)-ethyl]azacyclopentan-2-one (HPE-101),[9] glycerol monooleate,[10] 6-ketocholestanol (2% w/w)[11] and oleic acid.[12] Most reports examining the influence of chemical permeation enhancers on topical delivery of ALA have not studied a range of enhancer concentrations but have arbitrarily selected one concentration. However, many permeation enhancers, such as DMSO, act in a concentration-dependent manner. Clearly, there is a need to examine more than one concentration to determine enhancer efficacy.

The aim of this study was to incorporate several chemical penetration enhancers at various loadings into a patch-based delivery system. Pressure-sensitive[13,14] and bioadhesive patches[15,16] have been shown to offer a number of advantages over conventional semi-solid preparations. Patches are easy to apply, are retained at the site of administration, target drug delivery to the site of application and can be tailored to deliver a known amount of drug per unit area. Importantly, bioadhesive systems have the added benefit of adhering particularly well in moist environments, such as the lower female reproductive tract.

ALA penetration into porcine tissue from bioadhesive patches containing enhancers was examined *in vitro*. In addition, to gain an insight into the time-dependent nature of ALA penetration, drug permeation across excised mouse skin was also determined *in vitro*. Fluorescence spectrometry was used to determine ALA-induced PpIX fluorescence in the murine model *in vivo*. PpIX fluorescence was measured as a function of time following removal of the formulation. The effect of incorporating permeation enhancers into the patch was assessed by measuring ALA-induced PpIX fluorescence, both at the site of application and on the opposite flank of the animal.

Materials and Methods

Chemicals

5-Aminolevulinic acid hydrochloride salt was obtained from Crawford Pharmaceuticals (Milton Keynes, UK). Tripropyleneglycol methyl ether (Dowanol TPM) and DMSO were supplied by Sigma Aldrich (Dorset, UK). Tissue-TEK tissue embedding fluid was provided by Sakura Finetech Europe B.V. (Zoeterwade, The Netherlands). NCS-II tissue solubilizer was obtained from Amersham Biosciences (Buckinghamshire, UK). Ultima Gold liquid scintillation cocktail was obtained from PerkinElmer Life Sciences (Beaconsfield, Buckinghamshire, UK). Gantrez AN-139, a copolymer of poly(methyl vinyl ether) 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). Labrafac CC, Labrafac PG and Labrafil M1944CS were supplied by Gattefossé (UK) Ltd (Berkshire, UK). Labrafac CC is mixture of triglycerides of caprylic acid ($C_8H_{16}O_2$) and capric acid ($C_{10}H_{20}O_2$).[17] Labrafac PG is a mixture of propylene glycol diesters of saturated fatty acids, mainly caprylic acid and capric acid.[18] Labrafil M1944CS is a well-defined mixture of mono-, di- and triglycerides and mono- and di-fatty acid esters of polyethylene glycol.[19] Sevoflurane was obtained from Abbot (Germany). Radiolabelled 5-aminolevulinic acid solution, 3.7 MBq/ml was supplied by PerkinElmer Life Sciences. All other chemicals were of analytical reagent grade.

Preparation of ALA-loaded bioadhesive patches

Bioadhesive patches were prepared using a casting method from aqueous blends containing 20% w/w PMVE/MA and 10% TPM, as described previously.[15] Briefly, the required weight of PMVE/MA was added to water and stirred vigorously. The mixture was then heated and maintained between 95 and 100°C until a clear solution was formed. Upon cooling, the required amount of TPM was added and the casting blend adjusted to the final weight with water. Due to the increasing chemical instability of ALA at elevated pH,[20] the blend pH was not adjusted and, therefore, was around pH 2.

To the appropriate mass of blend, 158.27 mg ALA was added, giving each patch a constant ALA loading of 19 mg/cm^2. For formulations containing ^{14}C ALA, the radiolabelled source was withdrawn using a micropipette and added to the gel with stirring. Sufficient radioactivity was added to give approximately 5.9×10^5 disintegrations/min in each cm^2 of patch. Finally, 1.62 g of the blend was poured into a purpose-made poly(vinyl chloride) (PVC) mould of dimensions 45 × 12 mm, mounted on a glass support with a covering of siliconised release liner. For skin penetration studies, where relatively large circular films were required to fit into the donor compartment of the Franz cell, the desired amount of aqueous blend was poured into custom-made circular silicone moulds of the required surface area. Moulds were placed on a levelled surface to allow the blend to spread evenly across the area of the mould. The cast blend was dried under a constant air flow at 25°C.

Films were removed from the mould by simply peeling the release liner with attached film off the base of the mould. Bilaminar bioadhesive patches were prepared by attaching, with the aid of gentle pressure, the exposed side of the films containing ALA to equivalent areas of PVC backing films. For protection, the release liner was allowed to remain with its siliconised side attached to what had now become the release surface of the formed patch.

Patches loaded with 19 mg/cm^2 ALA and containing permeation enhancers were prepared by replacing water in the aqueous blend with equal weights of the permeation enhancers. Patches were prepared from blends containing 2, 5, 10, 15 and 20% w/w concentrations of DMSO, Labrafac CC, Labrafac PG and Labrafil M1944CS. For ^{14}C ALA radiolabelled formulations containing permeation enhancers, the radiolabel was added as described above in each case.

Skin

Full-thickness neonatal porcine skin was obtained from stillborn piglets and immediately (<24 h after birth) excised and frozen in liquid nitrogen vapour. Skin was then stored in aluminium foil at –20ºC. Female 7–8 week Balb/c athymic nude mice (20–25 g) were obtained from the animal department at the Royal Victoria Hospital, Belfast, UK. Animals were maintained under controlled conditions of temperature (23 ± 1°C) and humidity (45–60% RH) and had free access to food and water until they were killed for skin harvesting. Mice were killed by aspiration of carbon dioxide and the skin carefully excised. Excess subcutaneous fat and connective tissue was carefully removed and the skin washed with phosphate-buffered saline, wrapped in aluminium foil and stored at –20°C until further use. All skin samples were used within 2 weeks of preparation.[21]

Bioadhesion measurements

The bioadhesion properties of all films were evaluated quantitatively using the texture analyser in tensile mode. Full-thickness, shaved, neonatal porcine skin was attached with cyanoacrylate adhesive to a lower platform. Film segments (1 cm^2) were attached to the probe of the texture analyser using double-sided adhesive tape. Adhesion was initiated by adding a defined amount of water (10 μl) over an exposed skin sample (1 cm^2) and immediately lowering the probe with attached film. Upon contact, a force of 5 N was applied for 30 s before the probe was moved upwards at a speed of 0.1 mm/s. Adhesion was recorded as the force required to detach the sample from the surface of the excised skin. Results are reported as the mean ± SD of five replicates.

Evaluation of static ALA distribution in porcine tissue

Owing to the difficulties associated in obtaining excised human skin, neonatal pig skin was used as an in-vitro model. Pig skin has comparable morphological and functional skin characteristics to human skin[22] and is generally considered to provide the best in-vitro model of all domestic animals.[23] A further reason for using pig skin is its increased thickness compared with rodent skin.[23] Hence, penetration studies could be performed down to a depth of approximately 2.5 mm.

The distribution of ALA in neonatal porcine skin after a 4-h application period was determined using a modified jacketed Franz-type diffusion cell as described previously.[24] The cell was positioned upright and the receptor compartment filled with degassed phosphate-buffered saline at pH 7.4. The compartment was thermostatically controlled at 37°C, yielding a skin surface temperature of approximately 32°C.[2] Tissue biopsies were obtained by removing cylinders of tissue, approximately 5 mm in diameter and 3 mm deep, using a proprietary dermatological punch (Stiefel Laboratories Ltd, High Wycombe, UK). The tissue sample was mounted in a purpose-built steel washer, 4 mm thick, with an orifice 4 mm in diameter machined through its centre (Figure 1a). The specimen was supported on top of a stainless steel filter support grid that was placed across the top of the Franz cell reservoir.

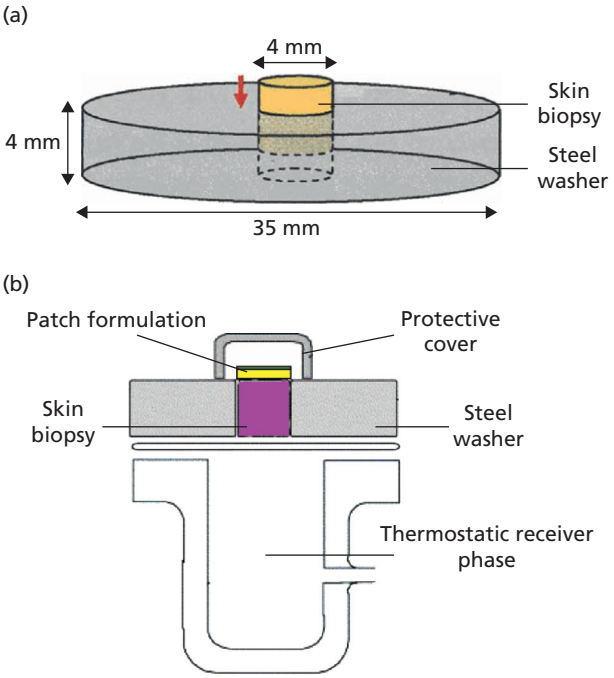

Figure 1 Modified Franz-type diffusion cell. (a) The skin biopsy was mounted in the 4 mm orifice of the steel washer with the epidermal layer facing upwards. (b) Cross-sectional representation of the modified Franz diffusion cell

At the beginning of the experiment, a disc-shaped sample of patch, 4 mm in diameter and containing both radiolabelled and unlabelled ALA, was applied to the epidermal surface of the skin biopsy with the aid of a 10-μl drop of water. A protective plastic cap, 7 mm in diameter, was placed over the assembly and secured with vacuum grease to prevent tissue dehydration (Figure 1b).

After a 4-h application period, the biopsy sample was removed from the washer and separated from the ALA patch. The surface of the specimen was carefully wiped with damp tissue to remove any traces of the formulation. The biopsy was then flash frozen in a liquid nitrogen atmosphere before tissue sectioning.

Microtome procedure

Cylinders of frozen tissue, pre-exposed to ALA-loaded patches, were mounted on the stage of a cryostatic microtome (Leica CM1900-1-1 cryostatic microtome; Leica Microsystems, Nussloch, Germany) using tissue embedding fluid. The microtome environment and stage operated at –25°C. Frozen 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 and five consecutive slices were taken and placed into the same scintillation vial. This procedure was repeated until the entire biopsy was sliced. The total depth of slicing into the tissue was approximately 2.5 mm. Occasionally, during tissue slicing, it was observed that the last few

cryosections were not uniform in diameter. When using the biopsy punch to remove tissue, the base of the excised tissue was rarely completely parallel to the skin surface. To negate these variations in tissue diameter at the base of the excised tissue, a mean depth of 1.875 mm was used as the region of interest.

Determination of radiolabelled ALA

Tissue slices containing radiolabelled ALA were dissolved in 1 ml NCS-II tissue solubiliser overnight at 37°C. Scintillation cocktail (5 ml) was added to each vial and mixed. To minimise chemiluminescence, vials were stored in darkness for 2 h before analysis. Samples were counted using a liquid scintillation analyser (Tri-Carb 2300 liquid scintillation analyser; Packard Instrument Company, Meridan, CT, USA) and the counts/min in each vial were determined over a 20-min period. An internally stored quench correction curve for ^{14}C converted the counts/min to disintegrations/min. Mean disintegrations/min values from three replicate experiments were converted into mean ALA concentrations. These ALA tissue concentrations were plotted against the mean tissue depth of each five-slice replicate. Mean penetration depths were the average depths for a given set of five consecutive slices, such that if 5 × 50-μm slices were taken from the top of a tissue sample, then the mean depth plotted against the mean ALA concentration would be 0.125 mm.

Evaluation of ALA penetration across excised full-thickness mouse skin

ALA penetration across excised full-thickness mouse skin was investigated *in vitro* using the conventional Franz cell model. For permeation studies, mouse skin was selected over pig skin for several reasons. Full-thickness pig skin is relatively thick and is difficult to mount on the Franz cell. Individual membranes can be prepared by various techniques,[25] however, these procedures are relatively complex and there is considerable scope for damaging the tissue.[2] In addition, in-vivo studies were going to be conducted using the mouse model. Therefore, gaining an insight into the time-dependent nature of ALA penetration across mouse skin *in vitro* was of particular interest.

The orifice diameter in both donor and receptor compartments was 1.0 cm, giving an effective cross-sectional area of 0.78 cm^2. Receptor compartment volumes, approximately 5 ml, were exactly determined by triplicate measurements of the weights of water they could accommodate. Account was taken of 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). The buffer was degassed by sonication before use. Murine skin was mounted onto the Franz cell with the dermal side facing downwards into the receptor compartment. Continuous stirring was provided by Teflon-coated stirring bars, rotating at 600 rev/min. Spring clips were used to hold the entire assembly together. The donor compartments were covered with laboratory film (Parafilm).

The penetration of ALA across excised murine skin was investigated by first removing 0.78-cm^2 bioadhesive patches from a custom-made PVC mould. Bioadhesive discs were attached to the skin in the donor compartments using 10 μl of deionised water. Using a long needle, samples (0.10 ml) were removed from the receptor compartment at defined time intervals (15, 30, 60, 120, 180, 240, 300, 360 and 420 min). This volume was immediately replaced using blank, pre-warmed buffer. Samples removed were analysed by fluorimetric high-performance liquid chromatography following derivatisation with acetyl acetone and formaldehyde, as described previously.[24] The mean flux (J) was calculated by Equation 1:

$$J = dM/dtS \quad (1)$$

where, M is the amount of ALA flowing through a unit crosssection, S, of skin in unit time, t, calculated from the linear region of each ALA penetration plot. The mean flux enhancement ratio (ER_J) was then simply the ratio of the flux obtained for the enhancer formulation compared with control.

In-vivo determination of ALA-induced protoporphyrin IX

Female Balb/c athymic nude mice (7–8 weeks old, 18–25 g) were obtained from the animal department of the Norwegian Radium Hospital (Oslo, Norway). Cages (three animals per cage) were housed in a scantainer maintained at a constant temperature (22°C) and humidity (60%). Food and bedding were sterilised and the mice were given potable water *ad libitum* in sterilised bottles. Inhalation anaesthesia using sevoflurane facilitated application of the formulations to the dorsal skin of mice. Generally, mice were awake within 5–10 min and returned to normal activity.

The siliconised release liner was removed from bioadhesive patches (surface area 1 cm^2) loaded with 19 mg/cm^2 ALA. Films were wetted with 10 μl of water and applied to normal dorsal skin of anaesthetised female nude mice. To prevent their dislodgment, patches were secured to the mouse by Scotch adhesive tape. Mice were kept in the dark to prevent PpIX photobleaching for 4 h, after which the dressings and formulation were completely removed and the area carefully washed with warm water and blotted dry with tissue paper.

Fluorescence was measured by means of a Perkin-Elmer LS50B luminescence spectrometer equipped with R3896 photomultiplier (Perkin-Elmer, Norwalk, CT, USA). The fluorescence measurements were carried out using a standard fiber-optic probe connected to the spectrometer. Fluorescence was measured at the formulation application site and on the contralateral flank as a function of time. The excitation wavelength was set at 407 nm, corresponding to the maximum of the soret band of the PpIX excitation spectrum in mouse skin, and fluorescence emission was measured at 635 nm. Emission spectra were measured to verify that the fluorescence signal originated mainly from PpIX. The excitation and emission slits were set at 10 and 15 nm, respectively. Scattered excitation light was removed from the detected light with a 515-nm cut-off filter. Before application of each formulation, background fluorescence (autofluorescence) of skin was recorded from each animal

and was subtracted from the fluorescence data. Fluorescence was measured up to 24 h after the start of application.

Imaging

The PpIX fluorescence of the mouse skin was visualised using a Kodak DCS 720× digital camera (Eastman Kodak, Harrow, UK). The fluorescence image was captured using a long-pass cut-off 630-nm filter in front of the camera objective and camera settings f2.8, 1/60, ISO3200 using blue excitation light (emission 350–400 nm, max 368 nm).

Statistical analysis

Comparison between patch formulations, in terms of ALA penetration into porcine skin, ALA delivery across murine skin and ALA-induced PpIX fluorescence *in vivo*, were made using the non-parametric multiple comparisons test, the Kruskal–Wallis test. Where significance was determined ($P < 0.05$), individual differences between treatments were identified using Dunn's post-hoc test.

Results

Bioadhesive films were prepared from aqueous polymeric blends containing 2, 5, 10, 15 and 20% w/w concentrations of DMSO, Labrafac CC, Labrafac PG and Labrafil M1944CS. It was found that when the concentration of enhancer was increased beyond 10% w/w, films were either excessively plasticised or did not form properly. Consequently, it was extremely difficult to biopsy film sections of the required size accurately. Factoring these observations into account, all further work was carried out with enhancer loadings of 2, 5 and 10% (w/w). Table 1 shows that the adhesive properties of preparations containing permeation enhancers were similar to the control patches.

Figure 2 illustrates that the concentration of ALA within the tissue shows depth dependence, with the concentration of ALA decreasing as progressive slices are taken down through the tissue biopsy. The highest concentrations were found in tissue slices closest to the site of application. Table 2 summarises the data relating to the penetration of ALA into excised neonatal porcine skin from each formulation examined. At 2% w/w enhancer loadings, Labrafac PG and Labrafil M1944CS both led to an increase in mean ALA concentration at 1.875 mm compared with the control patch (Table 2). In contrast, a reduction in ALA concentration was seen with patches containing 2% w/w DMSO and Labrafac CC compared with the control formulation. However, compared with control, these differences in delivery were

Table 1 Influence of permeation enhancer content in aqueous blends on the subsequent bioadhesion of cast films to neonatal porcine skin

Formulation	Adhesion (± SD) ; $n = 5$
Control (no enhancer)	0.964 ± 0.035
DMSO 2%	1.191 ± 0.284
DMSO 5%	0.9129 ± 0.320
DMSO 10%	0.891 ± 0.081
Labrafac CC 2%	1.256 ± 0.382
Labrafac CC 5%	1.162 ± 0.601
Labrafac CC 10%	1.277 ± 0.348
Labrafac PG 2%	0.578 ± 0.158
Labrafac PG 5%	0.821 ± 0.341
Labrafac PG 10%	1.261 ± 0.349
Labrafil M1944CS 2%	1.093 ± 0.185
Labrafil M1944CS 5%	1.398 ± 0.434
Labrafil M1944CS 10%	1.269 ± 0.549

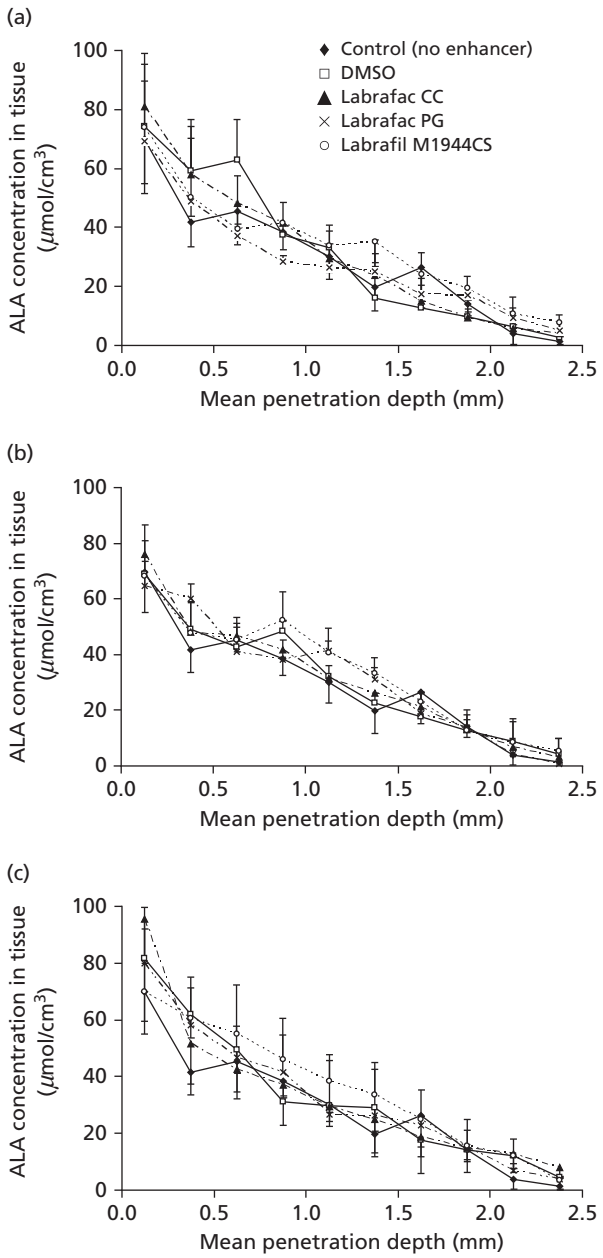

Figure 2 5-Aminolevulinic acid penetration across neonatal porcine skin. (a) Influence of 2% w/w loading of permeation enhancers on penetration of 5-aminolevulinic acid (ALA) into neonatal porcine skin. (b) Influence of 5% w/w loading of permeation enhancers on penetration of ALA into neonatal porcine skin. (c) Influence of 10% w/w loading of permeation enhancers on penetration of ALA into neonatal porcine skin. Patches contained 19 mg/cm^2 ALA and were applied for 4 h. Results represent means ± SD, $n = 3$

Table 2 Penetration of 5-aminolevulinic acid across excised porcine skin

Formulation	ALA concentration at 1.875 mm (μmol/cm^3) (± SD), n = 3	% of total drug loading released into tissue (± SD), n = 3
Control (no enhancer)	14.1 ± 3.95	6.43 ± 0.53
DMSO 2%	9.78 ± 2.72	6.95 ± 0.36
DMSO 5%	12.67 ± 5.53	6.81 ± 0.17
DMSO 10%	14.16 ± 7.97	7.34 ± 1.21
Labrafac CC 2%	9.50 ± 1.71	6.99 ± 0.75
Labrafac CC 5%	13.02 ± 6.50	6.93 ± 2.34
Labrafac CC 10%	14.76 ± 3.87	7.41 ± 0.69
Labrafac PG 2%	17.04 ± 6.41	6.27 ± 1.57
Labrafac PG 5%	13.65 ± 2.87	6.93 ± 0.40
Labrafac PG 10%	15.39 ± 5.55	7.25 ± 1.47
Labrafil M1944CS 2%	19.52 ± 4.11	7.45 ± 0.98
Labrafil M1944CS 5%	12.85 ± 7.25	7.44 ± 1.06
Labrafil M1944CS 10%	15.45 ± 9.55	7.95 ± 2.27

Patches were applied for 4 h and contained 19 mg/cm^2 5-aminolevulinic acid (ALA).

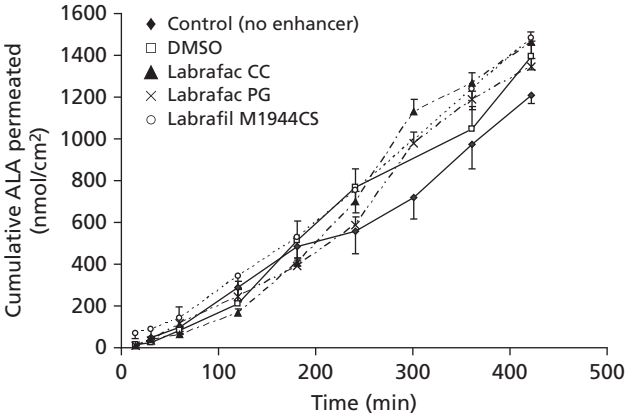

Figure 3 5-Aminolevulinic acid penetration across excised full-thickness mouse skin. Influence of 10% w/w loading of permeation enhancers on penetration of 5-aminolevulinic acid (ALA) across full-thickness excised mouse skin. Patches contained 19 mg/cm^2 ALA and were applied for 4 h. Results represent means ± SD, n = 3

not found to be significant. For example, control patches were associated with a mean concentration of 14.1 μmol/cm^3, which was not significantly different compared with the mean concentration of 9.5 μmol/cm^3 reported for the patches containing 2% w/w Labrafac CC ($P > 0.05$). When the permeation enhancer loading was elevated to 5% w/w, a reduction in the concentration of ALA at 1.875 mm was observed for all formulations containing enhancers compared with control (Table 2). In contrast, further increasing the enhancer loading up to 10% w/w resulted in an increase in ALA concentration at 1.875 mm for all four enhancers studied compared with control. However, ALA delivery from the 5 and 10% w/w enhancer films did not differ significantly compared with the control. For example, mean concentrations of 14.10 and 14.76 µmol/cm^3 were determined at 1.875 mm for the control patch and the patch containing 10% w/w Labrafac CC, respectively. Statistical analysis revealed that this difference was not significant ($P > 0.05$).

Table 2 also details the total percentage of drug delivered to the tissue from each formulation tested. All formulations that contained a penetration enhancer delivered increased amounts of ALA compared with the control ALA film. However, these apparent increases in the amount of ALA delivered were not found to be significant. For example, the percentage of total ALA loading delivered from the control patch was 6.43%, which was not significantly different compared with the 7.95% released from the formulation containing 10% w/w Labraphil M1944CS ($P > 0.05$). As can be appreciated from Table 2, no easily interpreted patterns were observed in terms of the percentage of ALA loadings released into porcine skin, regardless of the enhancer or enhancer concentration applied. The variability observed may be due to remnants of patches being harvested along with upper slices of some of the tissue samples. This assumption would also explain the extremely high ALA concentrations observed in the upper regions of some of the penetration profiles plotted.

ALA penetration across excised full-thickness mouse skin was determined as a function of time (Figure 3). Formulations were loaded with 19 mg/cm^2 ALA and contained 10% w/w penetration enhancer (DMSO, Labrafac CC, Labrafac PG and Labrafil M1944CS). The data obtained for each formulation resulted in similar shaped profiles, but with different levels of accumulated ALA. Table 3 shows that ALA flux increased in the presence of each enhancer (10% w/w loading). However, the enhancement seen compared with the control was only significant for films containing Labrafac CC ($P < 0.05$). The maximum ER_J was obtained for patches containing 10% w/w Labrafac CC. All formulations containing permeation enhancers delivered more ALA across murine skin than the control patch (Table 3). However, the increases in the percentage of drug loading delivered across murine skin were not significant compared with control ($P > 0.05$).

Typical fluorescence spectra of PpIX were observed in normal mouse skin after application of patches containing ALA (Figure 4a). Incorporation of chemical permeation enhancers did not influence the emission profile (Figure 4b), indicating that fluorescence predominantly originates from PpIX.[26] For in-vivo PpIX accumulation studies, different concentrations (2, 5, 10% w/w) of each permeation enhancer in the bioadhesive patch (ALA loading of 19 mg/cm^2) were prepared. For each formulation, 1 cm^2 of patch was continuously applied for 4 h to normal mouse skin. PpIX fluorescence intensity was continuously monitored upon removal of the formulation for 20 h. The accumulation kinetics of PpIX followed a similar profile for all patch formulations. Following removal of the vehicle, PpIX fluorescence at the application site peaked at 6–7 h (Table 4) and reduced to baseline levels at 24 h. The only exceptions were the control patch and Labrafac CC 10% w/w, which exhibited peak fluorescence at 8 h. From Table 4, it can be seen that an increase in the maximum ALA-induced fluorescence (F_{max}), compared with the control formulation, was seen for the majority of patches containing permeation enhancers. However,these increases were not found to be significantly higher than the control patch ($P > 0.05$).

Table 3 Influence of 10% w/w loading of permeation enhancers on penetration of 5-aminolevulinic acid across full-thickness excised mouse skin

Formulation	Flux (nmol/cm^2 per h) (± SD), n = 3	r^2 value	Flux enhancement ratio	% of drug loading delivered after 7 h (± SD), n = 3
Control (no enhancer)	178.16 ± 35.34	0.973	-	1.06 ± 0.05
DMSO 10%	219.27 ± 23.28	0.979	1.23	1.13 ± 0.08
Labrafac CC 10%	271.53 ± 3.46	0.980	1.52	1.19 ± 0.05
Labrafac PG 10%	237.26 ± 9.17	0.981	1.33	1.09 ± 0.04
Labrafil M1944CS 10%	231.39 ± 14.49	0.998	1.29	1.21 ± 0.16

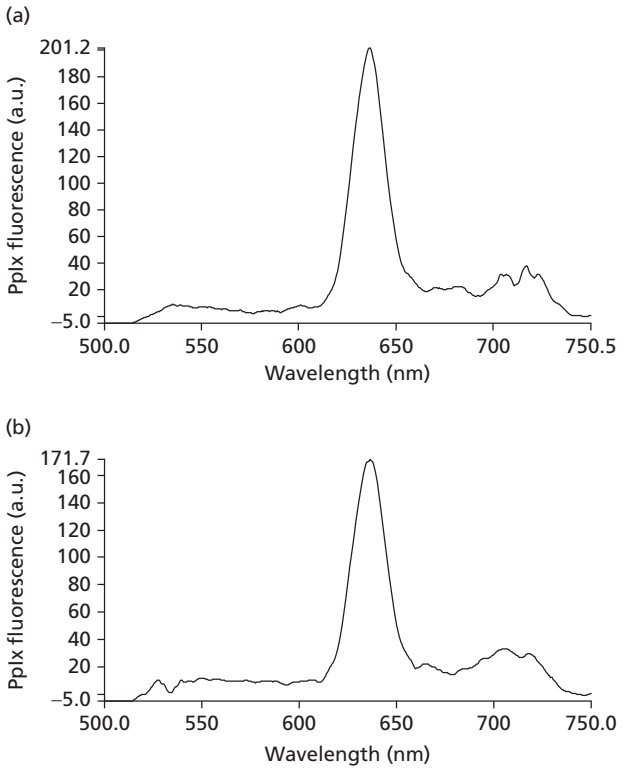

Figure 4 Fluorescence spectra of protoporphyrin IX in normal mouse skin after application of patches containing 5-aminolevulinic acid. (a) Typical emission spectrum of protoporphyrin IX (PpIX) after a 4-h application of a patch containing 19 mg/cm^2 5-aminolevulinic acid (ALA) (excitation wavelength 407 nm). (b) Typical emission spectrum of PpIX after a 4-h application of a patch containing 19 mg/cm^2 ALA and loaded with 2% w/w Labrafil M1944CS (excitation wavelength 407 nm)

PpIX fluorescence was also measured on the opposite flank to that where the formulation was applied. Table 5 shows that F_{max} values were much lower on the contralateral flank than those seen at the area of application (Table 4). The time at which the F_{max} was observed on the opposite flank was approximately the same as for the site of application. With the exception of formulations containing 5% w/w Labrafac PG and 5% w/w Labrafil M1944CS, an increase in F_{max} was seen with patches containing permeation enhancers. However, these increases were not significantly greater than control ($P > 0.05$).

Figure 5 shows PpIX fluorescence under UV illumination of the application site and opposite flank, respectively, 2 h after removal of a bioadhesive patch containing 2% w/w Labrafil M1944CS. It can be clearly seen that fluorescence is localised to where the patch was initially applied and that no significant fluorescence can be observed on the opposite flank.

Table 4 Influence of permeation enhancer loading on protoporphyrin IX accumulation at the site of application in normal murine skin

Formulation	Maximum protoporphyrin IX fluorescence recorded (a.u.) (± SD), n = 3	Time mean maximum protoporphyrin IX fluorescence observed (h)
Control (no enhancer)	216.1 ± 9.0	8
DMSO 2%	232.9 ± 19.7	6
DMSO 5%	225.4 ± 18.6	7
DMSO 10%	222.4 ± 27.8	7
Labrafac CC 2%	198.7 ± 13.7	6
Labrafac CC 5%	199.2 ± 22.3	6
Labrafac CC 10%	225.1 ± 7.31	8
Labrafac PG 2%	204.6 ± 5.2	7
Labrafac PG 5%	210.2 ± 30.8	6
Labrafac PG 10%	234.2 ± 18.8	7
Labrafil M1944CS 2%	221.8 ± 29.1	7
Labrafil M1944CS 5%	256.6 ± 32.9	7
Labrafil M1944CS 10%	260.2 ± 12.8	7

Patches were applied for 4 h and contained 19 mg/cm^2 5-aminolevulinic acid.

Discussion

High clearance rates have been obtained for the treatment of superficial lesions, such as Bowen's disease and actinic keratosis, using ALA photodynamic therapy. However, treatment of deeper lesions, for example nodular basel cell carcinoma, is less successful. This is thought to be due to poor penetration of ALA into skin lesions.[4,5] Over the past 15 years, there has been a concerted effort to investigate and develop strategies for enhancing the topical penetration of ALA into skin. Approaches that have been studied include the use of ion pairing,[27] laser ablation,[28] jet injection,[29] microneedles[30] and iontophoresis.[31] However, one of the most commonly employed enhancement strategies has been the use of chemical permeation enhancers.

The most widely studied permeation enhancer in the field of ALA photodynamic therapy is DMSO, which was shown to enhance PpIX production in cells in culture.[32] Malik *et al*.[5] examined the influence of 2% w/w DMSO in an ALA cream (20% w/w) *in vivo*. The authors reported a

Table 5 Influence of permeation enhancer loading on protoporphyrin IX accumulation in normal mouse skin on the opposite flank

Formulation	Maximum protoporphyrin IX fluorescence recorded (a.u.) (± SD), $n = 3$	Time mean maximum protoporphyrin IX fluorescence observed (h)
Control (no enhancer)	14.5 ± 2.7	6
DMSO 2%	16.7 ± 4.8	6
DMSO 5%	16.5 ± 3.7	6
DMSO 10%	19.8 ± 3.7	6
Labrafac CC 2%	17.4 ± 2.7	5
Labrafac CC 5%	14.9 ± 6.5	6
Labrafac CC 10%	15.2 ± 4.2	8
Labrafac PG 2%	14.6 ± 8.5	5
Labrafac PG 5%	12.9 ± 3.2	7
Labrafac PG 10%	17.2 ± 1.3	7
Labrafil M1944CS 2%	17.2 ± 6.8	6
Labrafil M1944CS 5%	13.2 ± 6.6	5
Labrafil M1944CS 10%	17.3 ± 3.0	5

Patches were applied for 4 h and contained 19 mg/cm^2 5-aminolevulinic acid.

modest increase in PpIX production when DMSO was included in the formulation. In this work, the authors noted that DMSO may act either as an enzyme inducer in the haem biosynthetic pathway or as a conventional permeation enhancer. More recently, other permeation enhancers, such as 6-ketocholestanol,[11] HPE-101[9] and oleic acid[12] have been examined. To date, all of these studies have utilised liquid or semi-solid vehicles, such as creams[5,12,33–36] and solutions.[4,37,38] In addition, many of the studies examining the effect of permeation enhancers on ALA delivery to date have investigated only one concentration of permeation enhancer.[4,5,9,34,35,37–39] However, the effect of many penetration enhancers is known to be concentration-dependent.[40, 41]

The majority of studies to date have shown limited effectiveness of penetration enhancers on the topical delivery of ALA. However, Steluti *et al.*[10] reported an approximate 75-fold increase in ALA flux across full-thickness excised mouse skin using the enhancer glycerol monooleate (20% w/w). Using this formulation, the authors claimed that almost 50% of the applied dose penetrated the mouse skin within 12 h. When tested *in vivo*, the 20% w/w glycerol monooleate formulation did not induce significantly higher levels of PpIX compared with the control vehicle. However, a significant increase in PpIX was seen using 10% w/w glycerol monooleate, compared with control. Grüning *et al.*[42] reported enhanced delivery of ALA across excised stratum corneum using a semi-solid liquid crystalline system. The authors attributed the higher flux values to the relatively high concentrations of isopropyl alcohol and surfactants within the formulation, which were thought to act as permeation enhancers.

The present study aimed to incorporate several permeation enhancers into a novel bioadhesive patch-type delivery system at different loadings. This patch has previously been shown to overcome many of the limitations associated with the use of conventional semi-solid and liquid preparations[16,43] and has been used in the successful photodynamic therapy of extra-mammary Paget's disease and lichen sclerosus of the vulva.[44,45]

The permeation enhancers studied were DMSO, Labrafac CC, Labrafac PG and Labrafil M1944CS. Although DMSO has been the most widely studied permeation enhancer with regards to the topical delivery of ALA to date, few studies have examined its concentration-dependent effect. Labrafac CC, Labrafac PG and Labrafil M1944CS are commercial permeation enhancers that have recently been investigated for the topical delivery of ALA methylester.[46] Importantly, these compounds are associated with low cutaneous toxicity.[17] Bioadhesive patches prepared using relatively low concentrations, up to 10% w/w, of each enhancer formed relatively well. However, inclusion of higher levels of penetration enhancers in the bioadhesive patch was not possible in this study due to their ability to excessively plasticise the bioadhesive matrix. Consequently, it was found that either removing these films from the mould or taking biopsies was very difficult. Not only was it considered impossible to sample films accurately with high loadings of enhancers, but such films would be unacceptable to patients and clinicians.

Depth penetration studies were carried out using excised neonatal porcine skin. The concentration of ALA determined at depths such as 1.875 mm can be compared with photodynamic therapeutic doses used in cell culture work. Moan *et al.*[47] showed that upon irradiation, 90% of cells could be killed with

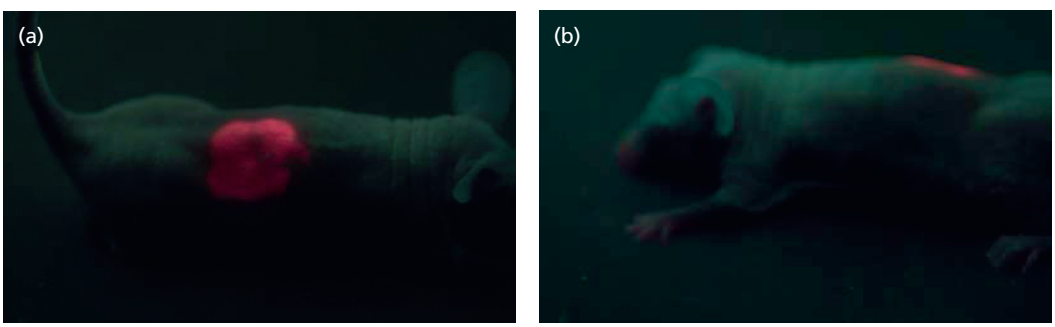

Figure 5 Protoporphyrin IX fluorescence under UV illumination of the application site and opposite flank after removal of a bioadhesive patch. View through a long-pass cut-off 515-nm filter under UV illumination of application site 2 h after removal of a bioadhesive patch containing 2% w/w Labrafil M1944CS. (a) Bioadhesive patch loaded with 2% w/w Labrafil M1944CS. (b) The contralateral flank

1 μmol/cm^3 ALA using the WiDr cell line. In similar work by Berg *et al.*,[48] Chinese hamster lung fibroblasts of the line V79 were exposed to 0.025 μmol/cm^3 ALA and illuminated for 15 min giving a 0.5 surviving fraction. Table 2 shows that the concentrations achieved at 1.875 mm exceeded these values by orders of magnitude using the control patch loaded with 19 mg/cm^3 ALA. Therefore, the amount of ALA present at this depth is in excess of that required in tissue culture media for PpIX production and subsequent cell death. Nodular basal cell carcinomas of depths greater than 2 mm are not uncommon.[49] However, due to the limited thickness of the tissue biopsies, ALA penetration beyond this could not be determined. The incorporation of permeation enhancers did not significantly increase the amount of ALA reaching the lower levels of the porcine skin ($P > 0.05$). Before performing in-vivo studies, skin permeation studies were performed *in vitro* to provide an insight into the time-dependent nature of ALA delivery across murine skin. Only patches containing 10% w/w Labrafac CC delivered significantly more ALA across murine skin than control patches ($P < 0.05$).

In-vivo studies were found to strongly agree with in-vitro results. Although there was a modest increase in fluorescence seen with some enhancer-containing patches, the increases were not significant ($P > 0.05$) and were poor compared with other enhancement strategies. For example, pretreating murine skin with microneedle arrays has been shown to almost double PpIX levels at low ALA loadings.[30] PpIX levels on the contralateral flank were significantly lower than those recorded at the site of application. There was no significant difference between the control patch and formulations that contained permeation enhancers. Figure 5 illustrates how the patch restricts PpIX fluorescence to the site of application. This is in contrast to the semi-solid ALA preparations, where PpIX fluorescence on the opposite flank of mice can be over 50% of that found at the application site.[36] The site of action of chemical permeation enhancers is thought to be primarily the stratum corneum. Enhancers are thought to cause disruption of the lipid bilayers, interact with intracellular protein and/or improve drug partitioning into the stratum corneum. ALA is a highly water soluble drug (log P_{ow} = –1.5) and is a zwitterion at physiological pH.[3] Consequently, the transcellular and shunt pathways are likely to be important routes for ALA penetration across skin over commonly used clinical application times, for example 4–6 h. Although the surface area occupied by hair follicles and sweat glands is small (typically 0.1% of the skin's surface area), it is considered to be an important pathway for polar molecules and ions,[50] particularly over short periods of time. Furthermore, appendages represent an invagination of the epidermis, extending deep into the dermis, hence providing an increased surface area for drug absorption.[51]

A recent study by Essa *et al.*[52] provides strong evidence that shunt routes may be a significant permeation pathway for very hydrophilic drugs. The authors examined the passive flux of the small hydrophilic compound mannitol (181.2 Da, log P_{ow} –2.47) using the human skin sandwich model. This model utilises a top sheet of stratum corneum, which is positioned on top of an epidermal membrane. It is assumed that the upper horny layer will block all of the available shunt routes in the lower epidermal membrane. Consequently, drug diffusion will be via routes other than the appendageal pathways. The authors reported that no mannitol penetrated across the skin sandwich for up to 10 h. However, when only a single sheet of epidermis was used, mannitol was detected in the receiver compartment within just 15 min, and levels increased in a linear fashion up to 10 h. This study clearly demonstrates that for small polar molecules, the shunt route plays a significant role in skin penetration.

If the appendageal route is the significant pathway for ALA penetration across skin then it would explain the findings seen in this study. Penetration enhancers are thought to act principally on the stratum corneum.[53,54] However, if ALA penetrates skin principally via the shunt route, then disruption of the stratum corneum may not significantly enhance its flux. Indeed, Koyama *et al.*[55] investigated the influence of d-limonene and oleic acid on the penetration of mannitol, mercaptopurine and butylparapen *in vitro.* The permeation enhancers were shown to promote penetration of the lipophilic drugs mercaptopurine and butylparapen. However, little effect was seen on mannitol penetration.

Conclusions

For the first time, the incorporation of penetration enhancers into a novel patch-based system for the delivery of ALA has been investigated *in vitro* and *in vivo.* Although this study has shown that enhancers did not improve the delivery of ALA from the patch, these agents may play a more significant role in the delivery of more lipophilic prodrugs of ALA. For example, the hexyl-ester of ALA has a log P_{ow} of 1.8 and is likely to navigate the stratum corneum primarily via the intercellular pathway. Currently, we are examining if agents that disrupt this pathway may be effective for improving the topical delivery of more lipophilic ALA prodrugs into the skin.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

References

1. Fritsch C *et al.* Photodynamic therapy in dermatology. *Arch Dermatol* 1998; 134: 207–214.
2. Williams AC. *Transdermal and Topical Drug Delivery.* London: Pharmaceutical Press, 2003.
3. Uehlinger P *et al.* 5-Aminolevulinic acid and its derivatives: physical chemical properties and protoporphyrin IX formation in cultured cells. *J Photochem Photobiol B* 2000; 54: 72–80.
4. Casas A *et al.* The influence of the vehicle on the synthesis of porphyrins after topical application of 5-aminolaevulinic acid. Implications in cutaneous photodynamic sensitization. *Br J Dermatol* 2000; 143: 564–572.

5. Malik Z *et al*. Topical application of 5-aminolevulinic acid, DMSO and EDTA: protoporphyrin IX accumulation in skin and tumours of mice. *J Photochem Photobiol B* 1995; 28: 213–218.
6. Roberts DJH *et al*. Tumour vascular shutdown following photodynamic therapy based on polyhaematoporphyrin or 5-aminolevulinic acid. *Int J Oncol* 1994; 5: 763–768.
7. Barry BW. Mode of action of penetration enhancers in human skin. *J Control Release* 1987; 6: 85–97.
8. Goodman M, Barry BW. Lipid-protein partitioning theory of skin enhancer activity: finite dose technique. *Int J Pharm* 1989; 57: 29.
9. van den Akker JT *et al*. Topical application of 5-aminolevulinic acid hexyl ester and 5-aminolevulinic acid to normal nude mouse skin: differences in protoporphyrin IX fluorescence kinetics and the role of the stratum corneum. *Photochem Photobiol* 2000; 72: 681–689.
10. Steluti R *et al*. Topical glycerol monooleate/propylene glycol formulations enhance 5-aminolevulinic acid in vitro skin delivery and in vivo protoporphyrin IX accumulation in hairless mouse skin. *Eur J Pharm Biopharm* 2005; 60: 439–444.
11. Auner BG *et al*. Influence of 6-ketocholestanol on skin permeation of 5-aminolevulinic acid and evaluation of chemical stability. *J Pharm Sci* 2004; 93: 2780–2787.
12. Pierre MB *et al*. Oleic acid as optimizer of the skin delivery of 5-aminolevulinic acid in photodynamic therapy. *Pharm Res* 2006; 23: 360–366.
13. Fauteck JD *et al*. Fluorescence characteristics and pharmacokinetic properties of a novel self-adhesive 5-ALA patch for photodynamic therapy of actinic keratoses. *Arch Dermatol Res* 2008; 300: 53–60.
14. Lieb S *et al*. Self-adhesive thin films for topical delivery of 5-aminolevulinic acid. *Eur J Pharm Biopharm* 2002; 53: 99–106.
15. McCarron PA *et al*. Design and evaluation of a water-soluble bioadhesive patch formulation for cutaneous delivery of 5-aminolevulinic acid to superficial neoplastic lesions. *Eur J Pharm Sci* 2006; 27: 268–279.
16. McCarron PA *et al*. Evaluation of a water-soluble bioadhesive patch for photodynamic therapy of vulval lesions. *Int J Pharm* 2005; 293: 11–23.
17. Bugaj A *et al*. The effect of skin permeation enhancers on the formation of porphyrins in mouse skin during topical application of the methyl ester of 5-aminolevulinic acid. *J Photochem Photobiol B* 2006; 83: 94–97.
18. Labrafac PG data sheet. Saint-Priest, France: Gattefosse, 2004.
19. Labrafil M1944CS data sheet. Saint-Priest, France: Gattefosse, 2004.
20. Novo M *et al*. Chemical instability of 5-aminolevulinic acid used in the fluorescence diagnosis of bladder tumours. *J Photochem Photobiol B* 1996; 34: 143–148.
21. Narishetty ST, Panchagnula R. Transdermal delivery of zidovudine: effect of terpenes and their mechanism of action. *J Control Release* 2004; 95: 367–379.
22. Meyer W *et al*. The skin of domestic mammals as a model for the human skin, with special reference to the domestic pig. *Curr Probl Dermatol* 1978; 7: 39–52.
23. Bronaugh RL *et al*. Methods for in vitro percutaneous absorption studies. II. Animal models for human skin. *Toxicol Appl Pharmacol* 1982; 62: 481–488.
24. Donnelly RF *et al*. Pharmaceutical analysis of 5-aminolevulinic acid in solution and in tissues. *J Photochem Photobiol B* 2006; 82: 59–71.
25. Kligman AM, Christophers E. Preparation of isolated sheets of human stratum corneum. *Arch Dermatol* 1963; 88: 702–705.
26. Juzeniene A *et al*. Topical application of 5-aminolevulinic acid and its methylester, hexylester and octylester derivatives: considerations for dosimetry in mouse skin model. *Photochem Photobiol* 2002; 76: 329–334.
27. Auner BG *et al*. Influence of lipophilic counter-ions in combination with phloretin and 6-ketocholestanol on the skin permeation of 5-aminolevulinic acid. *Int J Pharm* 2003; 255: 109–116.
28. Fang JY *et al*. Enhancement of topical 5-aminolaevulinic acid delivery by erbium:YAG laser and microdermabrasion: a comparison with iontophoresis and electroporation. *Br J Dermatol* 2004; 151: 132–140.
29. Donnelly RF *et al*. Influence of solution viscosity and injection protocol on distribution patterns of jet injectors: application to photodynamic tumour targeting. *J Photochem Photobiol B* 2007; 89: 98–109.
30. Donnelly RF *et al*. Microneedle-mediated intradermal delivery of 5-aminolevulinic acid: potential for enhanced topical photodynamic therapy. *J Control Release* 2008; 129: 154–162.
31. Lopez RF *et al*. Iontophoretic delivery of 5-aminolevulinic acid (ALA): effect of pH. *Pharm Res* 2001; 18: 311–315.
32. Fujita H *et al*. Sequential activation of genes for heme pathway enzymes during erythroid differentiation of mouse Friend virus-transformed erythroleukemia cells. *Biochim Biophys Acta* 1991; 1090: 311–316.
33. De Rosa FS *et al*. A vehicle for photodynamic therapy of skin cancer: influence of dimethylsulphoxide on 5-aminolevulinic acid in vitro cutaneous permeation and in vivo protoporphyrin IX accumulation determined by confocal microscopy. *J Control Release* 2000; 65: 359–366.
34. Soler AM *et al*. Photodynamic therapy by topical aminolevulinic acid, dimethylsulphoxide and curettage in nodular basal cell carcinoma: a one-year follow-up study. *Acta Derm Venereol* 1999; 79: 204–206.
35. Harth Y *et al*. Modified topical photodynamic therapy of superficial skin tumours, utilizing aminolevulinic acid, penetration enhancers, red light, and hyperthermia. *Dermatol Surg* 1998; 24: 723–726.
36. van den Akker JT *et al*. Systemic component of protoporphyrin IX production in nude mouse skin upon topical application of aminolevulinic acid depends on the application conditions. *Photochem Photobiol* 2002; 75: 172–177.
37. Perotti C *et al*. Topical application of ALA and ALA hexyl ester on a subcutaneous murine mammary adenocarcinoma: tissue distribution. *Br J Cancer* 2003; 88: 432–437.
38. Casas A *et al*. ALA and ALA hexyl ester-induced porphyrin synthesis in chemically induced skin tumours: the role of different vehicles on improving photosensitization. *Br J Cancer* 2001; 85: 1794–1800.
39. Ziolkowski P *et al*. Enhancement of photodynamic therapy by use of aminolevulinic acid/glycolic acid drug mixture. *J Exp Ther Oncol* 2004; 4: 121–129.
40. Nokhodchi A *et al*. The enhancement effect of surfactants on the penetration of lorazepam through rat skin. *Int J Pharm* 2003; 250: 359–369.
41. Nokhodchi A *et al*. The effect of terpene concentrations on the skin penetration of diclofenac sodium. *Int J Pharm* 2007; 335: 97–105.
42. Grüning N, Muller-Goymann CC. Physicochemical characterisation of a novel thermogelling formulation for percutaneous penetration of 5-aminolevulinic acid. *J Pharm Sci* 2008; 97: 2311–2323.
43. Gilmore BF *et al*. In vitro phototoxicity of 5-aminolevulinic acid and its methyl ester and the influence of barrier properties on their release from a bioadhesive patch. *Eur J Pharm Biopharm* 2006; 63: 295–309.
44. Zawislak AA *et al*. Successful photodynamic therapy of vulval Paget's disease using a novel patch-based delivery system containing 5-aminolevulinic acid. *BJOG* 2004; 111: 1143–1145.
45. McCarron PA *et al*. Photodynamic treatment of lichen sclerosus and squamous hyperplasia using sustained topical delivery of

aminolevulinic acid from a novel bioadhesive. *Br J Dermatol* 2004; 151: 105–106.
46. Bugaj A *et al*. The effect of dimethyl sulfoxide, 1-[2-(decylthio-)ethyl-]azacyclopentane-2-one and labrafac on porphyrin formation in mouse skin during topical application of methyl 5-aminolevulinate; a fluorescence and extraction study. *Photodiagn Photodyn Ther* 2006; 3: 27–33.
47. Moan J *et al*. Photobleaching of protoporphyrin IX in cells incubated with 5-aminolevulinic acid. *Int J Cancer* 1997; 70: 90–97.
48. Berg K *et al*. The influence of iron chelators on the accumulation of protoporphyrin IX in 5-aminolaevulinic acid-treated cells. *Br J Cancer* 1996; 74: 688–697.
49. Kennedy JC *et al*. Photodynamic therapy (PDT) and photodiagnosis (PD) using endogenous photosensitization induced by 5-aminolevulinic acid (ALA): mechanisms and clinical results. *J Clin Laser Med Surg* 1996; 14: 289–304.
50. Dokka S *et al*. Dermal delivery of topically applied oligonucleotides via follicular transport in mouse skin. *J Invest Dermatol* 2005; 124: 971–975.
51. Agarwal R *et al*. The pilosebaceous unit: a pivotal route for topical drug delivery. *Methods Find Exp Clin Pharmacol* 2000; 22: 129–133.
52. Essa EA *et al*. Human skin sandwich for assessing shunt route penetration during passive and iontophoretic drug and liposome delivery. *J Pharm Pharmacol* 2002; 54: 1481–1490.
53. Trommer H, Neubert RH. Overcoming the stratum corneum: the modulation of skin penetration. A review. *Skin Pharmacol Physiol* 2006; 19: 106–121.
54. Williams AC, Barry BW. Penetration enhancers. *Adv Drug Deliv Rev* 2004; 56: 603–618.
55. Koyama Y *et al*. Comparative analysis of percutaneous absorption enhancement by d-limonene and oleic acid based on a skin diffusion model. *Pharm Res* 1994; 11: 377–383.