Macromolecular delivery of 5-aminolaevulinic acid for photodynamic therapy using dendrimer conjugates

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Abstract

Intracellular porphyrin generation following administration of 5-aminolaevulinic acid (5-ALA) has been widely used in photodynamic therapy. However, cellular uptake of 5-ALA is limited by its hydrophilicity, and improved means of delivery are therefore being sought. Highly branched polymeric drug carriers known as dendrimers present a promising new approach to drug delivery because they have a well-defined structure capable of incorporating a high drug payload. In this work, a dendrimer conjugate was investigated, which incorporated 18 aminolaevulinic acid residues attached via ester linkages to a multipodent aromatic core. The ability of the dendrimer to deliver and release 5-ALA intracellularly for metabolism to the photosensitizer, protoporphyrin IX, was studied in the transformed PAM 212 murine keratinocyte and A431 human epidermoid carcinoma cell lines. Up to an optimum concentration of 0.1 mmol/L, the dendrimer was significantly more efficient compared with 5-ALA for porphyrin synthesis. The intracellular porphyrin fluorescence levels showed good correlation with cellular phototoxicity following light exposure, together with minimal dark toxicity. Cellular uptake of the dendrimer occurs through endocytic routes predominantly via a macropinocytosis pathway. In conclusion, macromolecular dendritic derivatives are capable of delivering 5-ALA efficiently to cells for sustained porphyrin synthesis. [Mol Cancer Ther 2007;6(3):876–85]

Introduction

The use of 5-aminolaevulinic acid (ALA) for photodynamic therapy (PDT) has received considerable interest owing to the metabolism of ALA via the haem biosynthetic pathway to the photosensitizer protoporphyrin IX (PpIX). Following exogenous administration of 5-ALA, PpIX is generated intracellularly, which can then be activated by visible light for PDT treatment. The principal advantages of ALA-PDT are the short duration of skin photosensitivity and its efficacy using both topical and p.o. administration (1, 2). The main clinical application has been in the treatment of nonmelatomatous skin lesions, mainly for basal cell carcinoma using topical application, although Food and Drug Administration approval has only been granted for the treatment of actinic keratosis. PDT of high-grade dysplasia in Barrett's esophagus using p.o. ALA administration is another active area of investigation (3). However, due to the hydrophilic nature of ALA, ALA-PDT has been hampered by the rate of ALA uptake into neoplastic cells and its limited penetration into tissue. Much effort has therefore been made to overcome the restricted bioavailability of ALA, either by derivitization or by the use of different delivery vehicles, such as liposomes (4). Esterified ALA prodrug derivatives have been widely studied (5–12), in particular the methyl and hexyl ester derivatives, and approval has been granted for treatment of actinic keratosis and basal cell carcinoma using the methyl ester derivative in Europe and Australia. Despite this progress, it is widely recognized that further improvements are required in ALA delivery.

There is much interest in developing new macromolecular delivery systems for cytotoxic agents in cancer therapy to improve their tumor uptake (13, 14). In this work, the use of macromolecular drug delivery vehicles based on hyperbranched polymers known as dendrimers was investigated. Dendrimers are promising candidates for improved drug delivery (15–18) because, unlike conventional polymers, they are prepared by a stepwise synthetic procedure, leading to a highly regular branching pattern and well-defined architecture, with a strictly controlled number of functional groups on their periphery for conjugation with bioactive molecules. These terminals may also be used as solubilizing groups, targeting groups, or other moieties to tune the biological properties and control the fate of the drugs. The key feature of dendrimer-drug conjugates is the ability to achieve a high drug payload by attaching multiple drug molecules to the dendrimer, which may be classified according to size ranging from first up to larger seventh-generation dendrimers (19).

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Because ALA is a relatively small molecule, it is a suitable candidate for conjugation with a macromolecule, such as a dendrimer. In principle, a dendrimeric nanovehicle bearing a high ALA 'payload' can per molecule deliver a much higher quantity of ALA to cells. A preliminary investigation of dendritic ALA derivatives showed that they were capable of delivering ALA to cells for conversion into PpIX (20). The ALA residues were attached via ester linkages, which were then cleaved enzymatically within the cells to liberate the ALA. Several smaller dendritic ALA prodrugs have also been investigated recently to compare the effect of changes in the molecular structure (21, 22).

In this work, we have focused on 'second-generation' dendrimers containing 18 ALA residues for delivery of ALA. The dendrimer was constructed using a convergent synthetic approach with a tripodent aromatic core for attachment of polyamidoamine branches bearing multiple ALA molecules covalently conjugated through ester linkages and prepared as a water-soluble trifluoroacetic acid salt. The ability to enter cells and release ALA intracellularly for conversion to PpIX and the resulting phototoxicity were investigated in two cell lines.

Materials and Methods

Chemicals

The structures of ALA and the dendrimer containing 18 ALA residues coupled via ester linkages [1,3,5-Tris[*N*-(*N*-bis{*N*-[tris(5-aminolaevulinyloxymethyl)methyl]propionamido}propionamido)carbamido]benzene 18 trifluoroacetic acid (18m-ALA); molecular weight, 3,678.8] are shown in Fig. 1. 5-ALA was purchased from Sigma-Aldrich (Poole, United Kingdom). The synthesis of the dendrimer was carried out as published previously (20). 5-(*N*-ethyl-*N*-isopropyl)amirolide (EIPA) and *N*-ethylmaleimide (NEM) were purchased from Fisher Scientific (Loughborough, United Kingdom; ACROS).

Stability Analysis

To examine the stability of the dendrimer in buffered aqueous solution at pH 7.4, solutions of 18m-ALA were prepared at concentrations up to 1 mmol/L, which was the highest concentration used in the pharmacokinetic studies. Solutions were prepared in 0.1 mol/L sodium dihydrogenorthophosphate dissolved in D_2O [for subsequent ^1H nuclear magnetic resonance (NMR) analysis] adjusted to pH 7.4. The solutions were then monitored over a period of several hours at 37°C for any changes in absorbance resulting from chemical degradation using a Perkin-Elmer Lambda 16 UV/visible spectrophotometer (Beaconsfield, United Kingdom). The spectrophotometer was scanned between 200 to 400 nm at suitable time intervals to determine the wavelength where the greatest differences in absorbance took place, which was identified as 270 nm. The change in absorbance against time was recorded at 270 nm. Formation of a precipitate was noted in 1 mmol/L

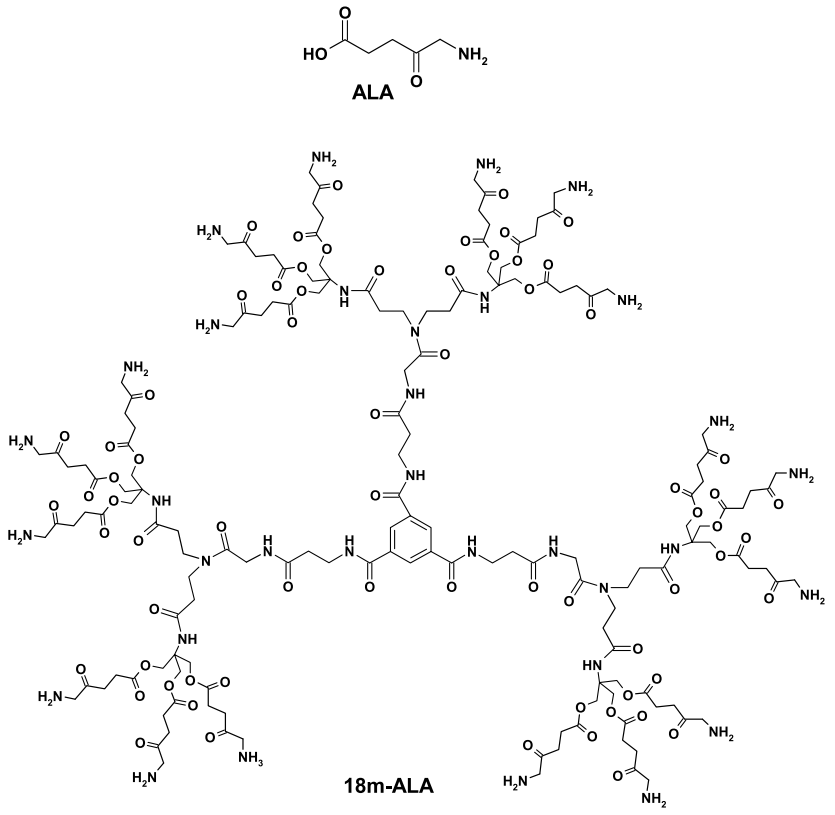

Figure 1. Molecular structures of 18m-ALA and ALA.

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18-ALA solutions. Both the supernatant and the precipitate [dissolved in deuterated DMSO (DMSO-d_6)] were then subjected to ^{1}H NMR analysis (EX270MHz, JEOL, Tokyo, Japan). Samples of supernatant were also analyzed for the presence of any free ALA released from the dendrimer using a Gilson reverse-phase high-pressure liquid chromatography system, eluted with 1% trifluoroacetic acid in methanol and water (ratio, 1:9).

Cell Culture

The tumorigenic PAM 212 cell line, which is derived from murine epidermal keratinocytes (donated by R. Groves, King's College, London, United Kingdom), was cultured in RPMI 1640 (Life Technologies Ltd., Paisley, United Kingdom) containing L-glutamine (0.02 mmol/L) and phenol red, supplemented with 10% FCS (First Link, Birmingham, United Kingdom) and penicillin/streptomycin (500 units/mL and 0.5 mg/mL; Life Technologies). The cells were routinely cultured at 37°C in a 5% CO_2/air humidified atmosphere until ~80% confluent. The human epidermoid carcinoma A431 cell line was obtained from the European Collection of Animal Cell Cultures. The medium used in this case was MEM (Invitrogen, Gibco Cell Culture Systems, Life Technologies) supplemented with 1% nonessential amino acids, gentamicin (50 mg/mL), 10% fetal bovine serum, and L-glutamine (0.02 mmol/L). The cells were cultured as described above for the PAM 212 cell line.

Fluorescence Pharmacokinetics

Cells were seeded into γ-sterilized 96-well plates (Orange Scientific, Triple Red Laboratory Technologies, Long Crendon, United Kingdom) at a density of ~5×10^4 cells per well for 48 h. After removing the culture medium, the wells were washed with PBS. The cells were incubated with freshly prepared solutions of ALA and ALA-containing prodrugs: 0.1 mL serum-free medium containing varying prodrug concentrations was added to a designated series of wells. Each plate contained control wells with cells but without added drug for determination of the background reading and reference wells containing cells incubated with the same ALA concentrations. For drug incubation, serum-free medium was used because serum is known to cause release of PpIX from cells, thus resulting in loss of the fluorescence signal (7).

The fluorescence signal from each well was measured with Perkin-Elmer LS 50B fluorescence spectrometer coupled to an automated plate reader (Perkin-Elmer) using 410 nm excitation and 635 nm emission wavelengths with slit widths set to 10 nm and an internal 530 nm longpass filter used on the emission side; spectral scans were recorded between 600 and 750 nm to check for presence of any porphyrins other than PpIX (21). The mean fluorescence was calculated after subtraction of the control values. This method of comparing ALA-induced PpIX kinetics corresponds closely with the study of Uehlinger et al. (23) Intensity calibrations were done using rhodamine B embedded in a Perspex disc as a standard.

Photodynamic Treatment

Cells were seeded into 96-well plates at a density of ~5×10^4 cells per well. Following incubation for 48 h, the cells were washed with PBS and 0.1 mL solutions containing each compound at varying concentrations (0.01, 0.05, 0.1, 0.5, and 1 mmol/L) were added to their designated wells for 4-h incubation. Each well plate contained six control wells without the compound and the compound at five different concentrations in sextuplicate. The plates were irradiated with a fluence of 5 J cm^{-2} using a LumiSource lamp (PCI Biotech, Oslo, Norway), which emits a uniform field of low power blue light over an area of 14 cm × 32 cm. Peak output is ~420 nm, which overlaps well with the porphyrin Soret band. Immediately following irradiation, the medium was replaced and cells were incubated for a further 24 h. Cell cytotoxicity was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay: cells were incubated with medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (1 mg/mL dissolved in full RPMI 1640) for 3 h. The insoluble end product (formazan derivative) was dissolved in 0.1 mL DMSO after removing the medium. Visible absorption was quantified at 570 nm using a 96-well plate reader (MR 700 Dynatech; Dynatech, Billingshurst, United Kingdom). The mean cell survival was calculated for each prodrug at every concentration tested and expressed as a percentage of control (incubated with the compounds but not irradiated) cell survival values. For determination of 'dark' toxicity of the compounds, well plates were prepared in the same manner as above without irradiation. The cells were incubated with the compounds for 3 h, washed, and, 24 h later, subjected to the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

Cellular Uptake Mechanism

Experiments were conducted using ALA and 18m-ALA exposed to PAM 212 cells for short periods to assess the internalization of the dendrimer. Cells were seeded in 96-well plates for short exposure to the compounds for periods of 15, 30, 45, and 60 min. Following incubation, the cells were washed thrice with PBS and incubated for a further period in serum-free medium to allow time for PpIX synthesis; measurements of porphyrin fluorescence were carried out up to 24 h later from the start of incubation with the compounds.

The possibility of endocytic mechanisms contributing to the cellular uptake of 18m-ALA was investigated using confocal microscopy and fluorescence spectroscopy. 18m-ALA was incubated with PAM 212 cells at low temperature (4°C) to suppress endocytosis or at 37°C for 1 h. Cells (1×10^4) were seeded into 35-mm glass-bottomed dishes (Iwaki, Takao, Japan) and cultured as described below for confocal microscopy. For PpIX fluorescence spectroscopy measurements using the spectrofluorometer, cells were seeded in 96-well plates (5×10^4) and incubated at 4°C or 37°C with fresh medium containing ALA or 18m-ALA for 1 h and washed thrice with cold PBS. For both imaging and spectroscopic measurements, incubation was continued at 37°C for a further 3 h to allow time for PpIX synthesis.

Confocal fluorescence microscopy was used to study the intracellular levels and localization of PpIX using excitation at 543 nm (Bio-Rad Radiance 2000/Olympus

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1X70; Bio-Rad, Hemel Hempstead, United Kingdom; Olympus, London, United Kingdom). Approximately 50 cells were seeded onto microscope coverslips (13 mm in diameter) and placed in circular Petri dishes (35 mm in diameter; Orange Scientific) 48 h before the experiment. After 48 h, they were washed once with PBS and inoculated with 2 mL of fresh medium containing different concentrations of ALA and 18m-ALA prodrugs and incubated for 3 h. Controls with cells incubated with medium alone were also tested for any autofluorescence. For more detailed examination of the uptake mechanism, the effects of the macropinocytosis inhibitor, EIPA, or the caveolae-mediated endocytosis inhibitor, NEM, were compared: the cells were prepared as described above for confocal microscopy and fluorescence spectroscopy measurements, then pretreated with 0.1 mmol/L EIPA for 60 min (24) or 1 mmol/L NEM for 2 min (25), and washed thrice with PBS. The cells were then incubated with ALA or 18m-ALA. ALA controls were included to show that the inhibitors did not interfere with the metabolism of ALA to PpIX. Images were obtained for cells after 3-h incubation with ALA or 18m-ALA.

Statistical Analysis

The results are displayed graphically or tabulated with error bars representing the SDs of quadruplicated measurements. Differences are considered to be significant using the unpaired t test with $P < 0.05$.

Results

Fluorescence Pharmacokinetics

The kinetics of porphyrin fluorescence induced by exposure to 18m-ALA and ALA in the PAM 212 and A431 cell lines are shown in Fig. 2. PpIX production in the PAM 212 cell line was found to be at least 50% higher than that observed using the A431 cell line at most concentrations for both ALA and 18m-ALA compounds. The kinetics of PpIX from 18m-ALA exhibited a dose-dependent response with porphyrin production peaking at low concentrations ranging between 0.01 to 0.1 mmol/L in both cell lines. Increasing porphyrin levels were observed with increasing incubation times from 2 to 24 h, and at 24 h, the PpIX fluorescence measured using 0.1 mmol/L 18m-ALA was 50% higher than found using 1 mmol/L ALA. A pronounced reduction in porphyrin generation was found at higher concentrations of 18m-ALA up to 1 mmol/L. However, the 18m-ALA dendrimer was clearly more efficient compared with ALA with respect to PpIX generation over the lower concentration range for all incubation periods.

Experiments were conducted using ALA and 18m-ALA exposed to PAM 212 cells for short periods to assess the rate of internalization of the dendrimer. Because each dendrimer contains 18 ALA molecules, a direct comparison was also made using an 18-fold higher ALA concentration (i.e., 18 × equimolar). In these experiments, PAM 212 cells were incubated with the compounds for set periods: 15 to 60 min. The concentration of dendrimer used was 0.1 mmol/L, which gave maximal PpIX fluorescence readings. The corresponding ALA concentration was 1.8 mmol/L,

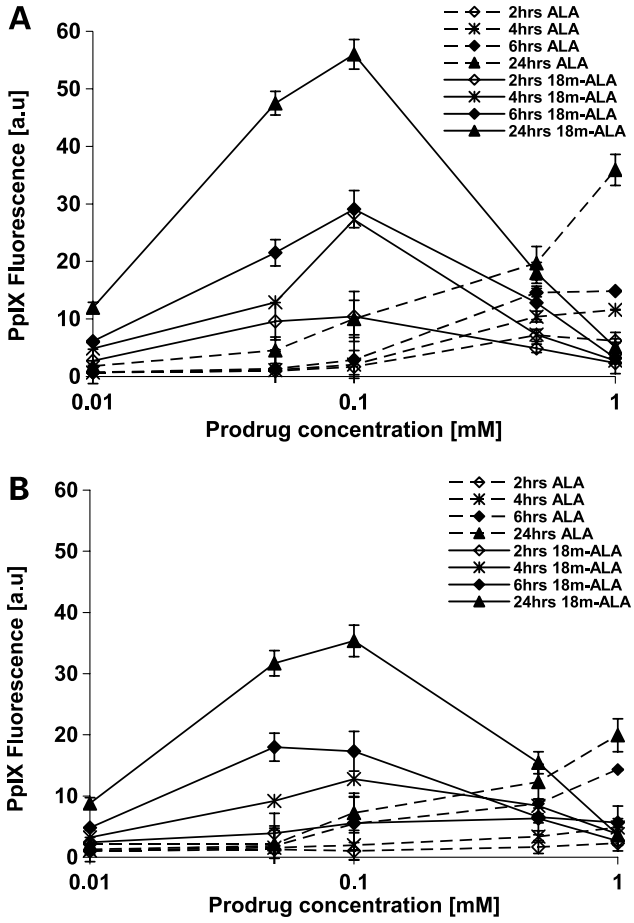

Figure 2. **A,** kinetics of PpIX fluorescence in PAM 212 cells. A time course study of fluorescence intensity measurements over 2-, 4-, 6-, and 24-h incubation times for concentrations of ALA or 18m-ALA ranging between 0.01 to 1 mmol/L. **B,** the same study of PpIX fluorescence using ALA and 18m-ALA in A431 cells.

which is below the threshold for any toxic effects. The cells were then washed to remove the compounds and incubated in fresh medium for a further period. Further incubation following removal of the compounds was necessary to metabolize the internalized molecules to PpIX. The results are presented in Fig. 3. Figure 3A shows results where the total incubation period (with the compounds plus the following interval without the compounds) was fixed at 4 h, and likewise in Fig. 3B, the total incubation period was fixed at 24 h. Using a 4-h measurement time point, comparable PpIX fluorescence intensities were observed using 18m-ALA at 0.1 mmol/L compared with incubation using ALA at 1.8 mmol/L for short exposure times from 15 to 60 min, and the porphyrin fluorescence levels increased in each case by approximately a factor of four with the increasing exposure time. Figure 3B however shows that with a 24-h measurement time point, the dendrimer yielded significantly higher PpIX levels than ALA: using 60-min exposure and the 24-h time point, the fluorescence levels were four times higher using the dendrimer

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compared with ALA. This divergence arises because the porphyrin fluorescence intensity induced using ALA is much lower at 24 h compared with 4 h, whereas with the dendrimer, comparable levels were found using the 4- and 24-h time points. Experiments were also carried out using intermediate time points of 2 and 6 h, which confirmed that peak porphyrin levels induced by ALA occurred at earlier time points: using 60-min exposure to ALA, the intensity observed at 6 h was 20.3 ± 0.9 a.u. compared with 4.1 ± 0.7 a.u. observed at 24 h. In contrast, there was a sustained increase in porphyrin levels induced by the dendrimer from 2 to 24 h. As discussed below, these results indicate that ALA is released gradually from the dendrimer.

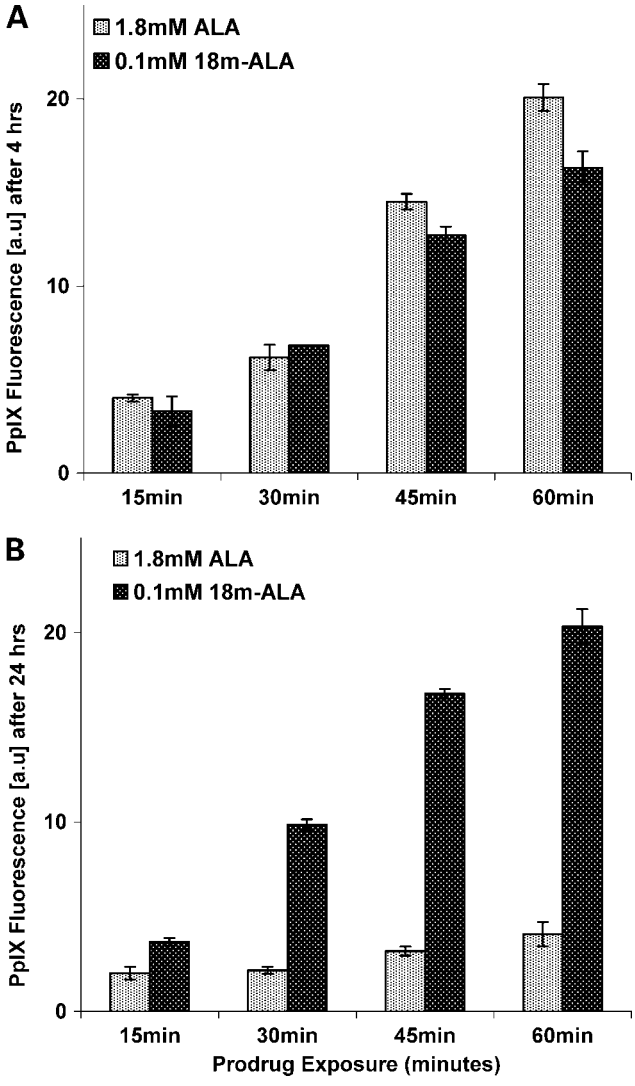

Figure 3. PpIX production in PAM 212 cells treated with 0.1 mmol/L 18m-ALA or 1.8 mmol/L ALA (i.e., 18 × equimolecular concentration). **A,** cells were exposed to the compounds for a range of times up to 60 min and then washed to remove the compound. In each case, the fluorescence reading was taken 4 h after the time point at which cells were first exposed to the compound. **B,** as for (**A**), but fluorescence readings were taken 24 h from the time point at which cells were first exposed to compounds.

Stability Evaluation

The chemical stability of the dendrimer in aqueous solution was analyzed using UV absorption spectrometry, NMR, and high-pressure liquid chromatography techniques. All of these assays showed that chemical degradation of the dendrimer at 37°C in buffered pH 7.4 aqueous solution took place over a period of hours, as is known to occur with ALA itself, although acidic aqueous stock solutions were stable. At the highest concentration studied of 1 mmol/L, precipitation to give a brownish residue was evident after 2-h storage. Both the supernatant and precipitate were analyzed, although the precipitate was only weakly soluble in deuterated DMSO (for ^{1}H NMR analysis). Analysis of the supernatant by NMR and high-pressure liquid chromatography confirmed that there was no release of ALA from the dendrimer. NMR spectra obtained from DMSO solutions of the precipitate were weak and no conclusions about the chemical composition of this material could be drawn, although its precipitated brownish appearance would be consistent with the formation of a polymeric degradation product.

Photodynamic Treatment

The phototoxicity after 4-h incubation with the ALA and 18m-ALA prodrugs was investigated in both cell lines over a range of concentrations. The percentages of cell survival with respect to control cells (without compounds) were calculated for various concentrations of investigated compounds and plotted as shown in Fig. 4A. 18m-ALA was more effective than ALA at low concentrations for both cell lines: <5% cell survival and 35% cell survival at a concentration of 0.05 mmol/L were observed in PAM 212 and A431 cells, respectively, compared with >90% cell survival using ALA in both cell lines. The 'dark' toxicity of the 18m-ALA was also assessed (i.e., their cytotoxicity without irradiation) as shown in (Fig. 4B); the data indicate that there was little toxicity after incubation with 18m-ALA at a concentration up to 0.2 mmol/L after 24-h incubation. The higher phototoxicity observed using the PAM 212 cell line is consistent with the higher porphyrin synthesis compared with A431 cells.

Internalization Mechanism of 18m-ALA Dendrimer

The role of endocytic pathways in the cellular uptake mechanism was investigated using low-temperature incubation with 0.1 mmol/L 18m-ALA at 4°C for 1 h, which should inhibit endocytosis, compared with incubation at 37°C; after incubation with the compounds, the cells were washed and incubated for a further 3 h at 37°C to allow time for PpIX synthesis. Figure 5A shows that significant reductions in porphyrin levels were observed over a range of concentrations using incubation at 4°C compared with 37°C. Confocal microscopic images of porphyrin fluorescence confirmed the reduction of PpIX at low temperature compared with 37°C. For examination of macropinocytosis or caveolae-mediated contributions to the cellular uptake mechanism, the macropinocytosis inhibitor, EIPA (24), and caveolae-mediated endocytosis inhibitor, NEM, were used (25); PpIX production induced by 18m-ALA after EIPA pretreatment was reduced by 90% to that without EIPA

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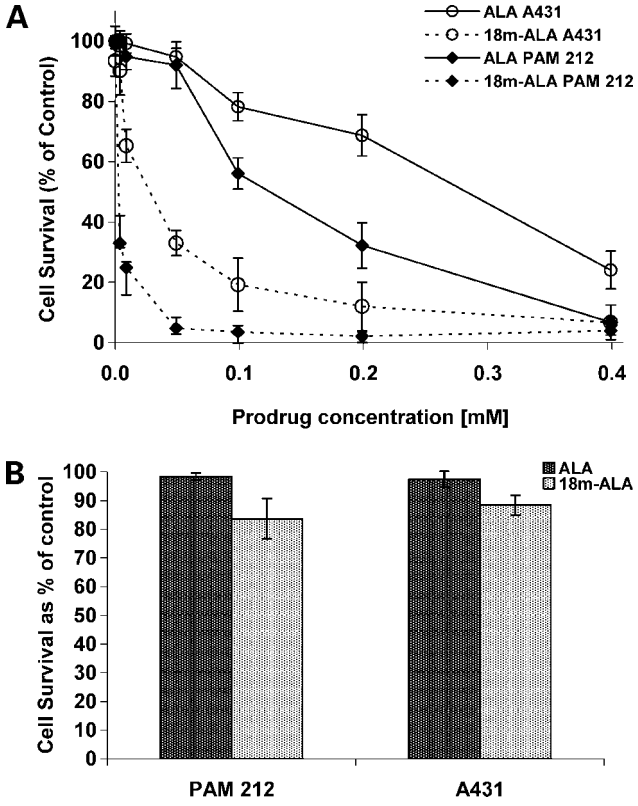

Figure 4. **A,** phototoxicity after incubation with ALA and 18m-ALA at a range of concentrations in A431 and PAM 212 cell lines assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cells were incubated with the compounds for 4 h and irradiated with blue light (5 Jcm^{-2}). **B,** dark toxicity measurements. Cells were incubated with 0.2 mmol/L ALA or 18m-ALA for 24 h in the dark without irradiation.

treatment (Fig. 5B), whereas PpIX generated by ALA was not affected with EIPA pretreatment. The fluorescence produced after pretreatment of cells with NEM and incubated with 18m-ALA was less affected: the PpIX fluorescence was ~17% lower, and no change was observed using ALA. Confocal microscopic images (Fig. 6A and B) also showed the reduction in PpIX fluorescence as a result of EIPA inhibition of 18m-ALA uptake but not after pretreatment with the caveolae inhibitor (NEM; Fig. 6C).

Discussion

A rapid increase in research on macromolecular drug conjugates has been reported in recent years due to the fact that many low molecular weight drug candidates are limited in their therapeutic value by solubility, poor bioavailability, and rapid elimination. New delivery technologies that could overcome the internalization barrier, including the use of dendrimers, are therefore being investigated (19). Improved targeting to solid tumors can also be achieved on conjugation of anticancer drugs to polymers (26), which is believed to arise from the increased permeability of tumor vasculature to macromolecules and limited lymphatic drainage (27–29).

In this work, the delivery of 5-ALA to cells for PDT using a macromolecular prodrug delivery system was investigated, with the aim of showing that a macromolecule bearing a high ALA payload can be delivered efficiently to cells and then undergo sustained enzymatic hydrolysis to liberate ALA for subsequent conversion to PpIX. The efficiency of 5-ALA induced PpIX generation using the prodrug concept is controlled by three independent processes: (*a*) the rate of drug uptake; (*b*) its rate of enzymatic conversion into PpIX, and (*c*) the enhanced retention at the application site (12). In this study, we investigated the properties of second-generation macromolecular dendritic carriers containing 18 residues of ALA. The structure of the dendrimer was designed to enable 18 ALA residues to be attached by controlled synthesis via ester linkages to multipotent aromatic core. Fluorescence spectroscopy was used to detect and quantify the porphyrin accumulation induced by the

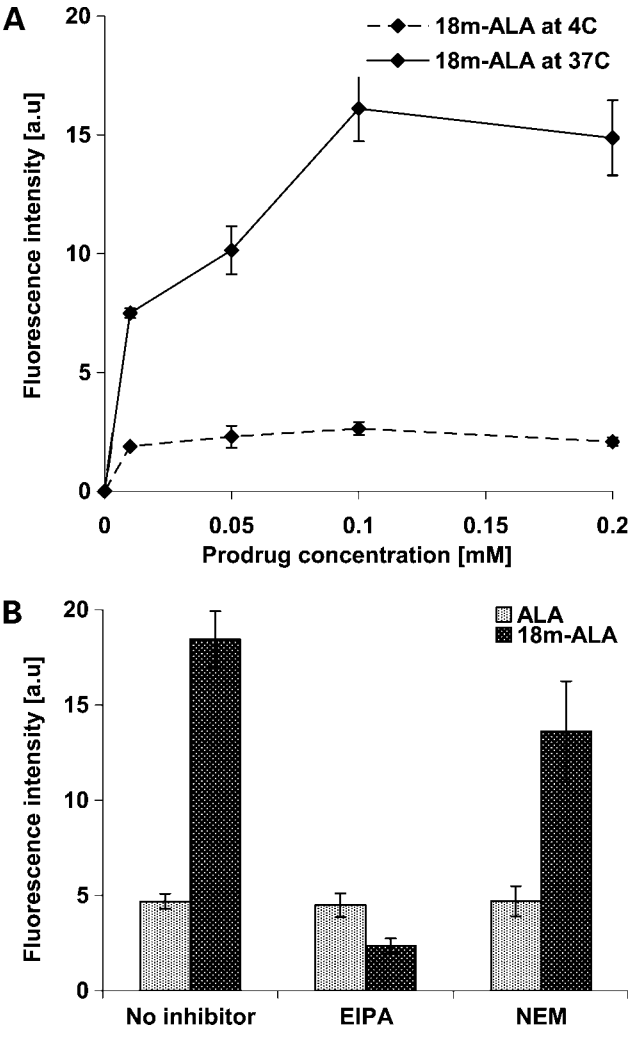

Figure 5. Cellular uptake mechanism. **A,** PAM 212 cells were exposed for 1 h to 18m-ALA at 4°C or 37°C, then washed, and incubated for a further 3 h. **B,** PpIX fluorescence produced by 0.1 mmol/L ALA or 18m-ALA incubated for 4 h in PAM 212 cells pretreated with EIPA or NEM inhibitors.

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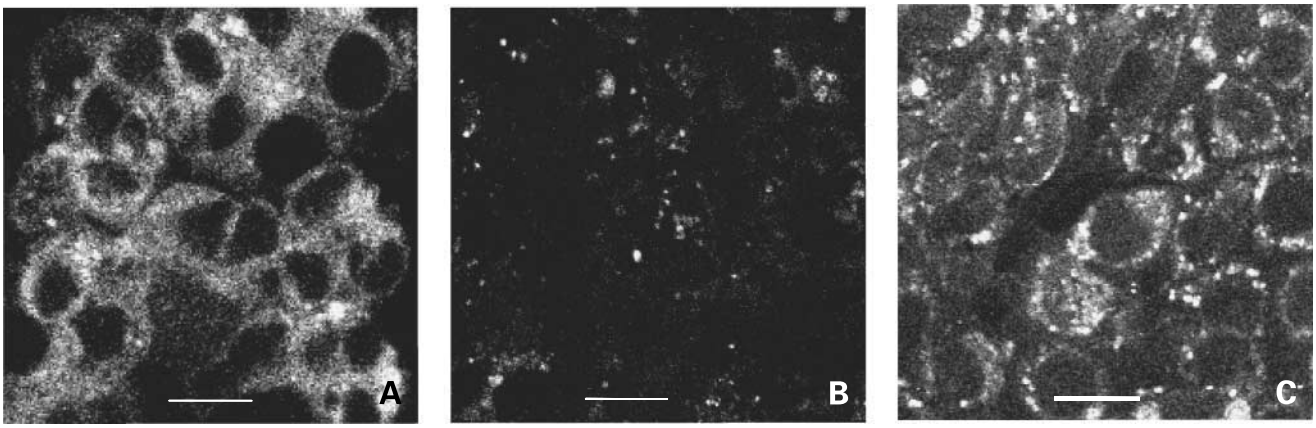

Figure 6. Confocal microscopy images of PpIX fluorescence in PAM 212 cells following incubation with 0.1 mmol/L 18m-ALA showing effect of EIPA and NEM inhibitors. **A,** cells were incubated with 18m-ALA only at 37°C for 3 h. **B,** cells were pretreated with EIPA and then incubated with 18m-ALA for 3 h at 37°C. **C,** cells were pretreated with NEM and then incubated with 18m-ALA for 3 h at 37°C. Bar, 8 μm.

dendrimer versus ALA in the transformed murine keratinocyte PAM 212 and the A431 human epidermoid carcinoma cell lines.

Porphyrin generation was found to be more efficient for both ALA and the dendrimer in the PAM 212 cell line. Previous work using comparing these cell lines with ALA (30) also showed higher porphyrin synthesis in the PAM 212 cells, which may in turn account for the higher porphyrin levels found using dendrimer delivery of ALA. Following incubation with equimolar doses, porphyrin generation after exposure to 18m-ALA was significantly higher compared with ALA at low concentrations between 0.01 to 0.1 mmol/L in both cell lines. This improvement must be due in part to the high ALA payload per dendrimer molecule assuming that there is efficient cleavage of the ALA residues, as discussed later. Following illumination of cells incubated with the dendrimer, cell survival was significantly reduced (Fig. 4A) compared with equimolar ALA, in good agreement with the pharmacokinetic studies. Higher phototoxicity was observed using both compounds in PAM 212 cell line than the A431 line, which is consistent with the higher porphyrin production observed in the PAM 212 cells.

The decrease in porphyrin generation observed at higher dendrimer concentrations is most likely due to chemical degradation of the dendrimer in the culture medium, based on the stability studies carried out in buffered aqueous solutions, in which we noted formation of a precipitated residue, possibly of polymeric composition. This degradation probably occurs through intermolecular reactions between ALA residues on different dendrimers resulting in polymerization, which would be accelerated at higher dendrimer concentrations; an intramolecular reaction may also be possible although sterically hindered. In previous work using smaller dendritic derivatives bearing three ALA residues (21), we observed a similar phenomenon that we proposed arises from intermolecular condensation reactions occurring between ALA residues attached to different dendritic molecules. This proposed mechanism stemmed from the well-known dimerization reactions that can occur for ALA and its esterified ALA derivatives at physiologic pH (4). Such reactions for the dendrimer could be inhibited by derivitization of the exposed ALA amino groups, although once internalized, the dendrimers should be stabilized following incorporation within acidic lysosomal vesicles through endocytosis, as discussed below. Other possible factors affecting the efficacy at high concentrations include potential toxic effects of the cleavage products. Another potential problem with molecular carriers bearing multiple ALA residues is acidification of the medium at higher concentrations (21). However, this effect was mitigated in the present study by administering the dendrimers as trifluoroacetic acid salts instead of strongly acidic hydrochloride salts.

Understanding the internalization mechanism of dendritic carrier of the prodrugs is fundamental to their use as delivery vectors. The cellular uptake of ALA has been studied by several groups and is mediated via active transport mechanisms, whereas passive diffusion is also significant for small ALA esters (31–33). Much larger molecules, such as ALA dendrimers, however, may be taken up through endocytic pathways. The molecular weight of the 18m-ALA dendrimer (excluding trifluoroacetic acid) is 3,678.8. To examine the contribution of endocytic processes on 18m-ALA uptake, we conducted experiments in PAM 212 cells at 4°C because all endocytic processes, clathrin-dependent and clathrin-independent endocytosis, micropinocytosis, and macropinocytosis, are blocked at low temperature (34, 35). Porphyrin production was severely suppressed when cells were incubated with 18m-ALA at 4°C compared with 37°C, which is consistent with endocytic uptake.

Several endocytic routes have been suggested for the cellular uptake of some macromolecules with similar structures and molecular size to our 18m-ALA dendrimer, such as arginine-rich peptides (24, 36) and polyamidoamine dendrimers (17). Macropinocytosis, which is a form of nonreceptor-mediated endocytosis, is one of the major

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pathways for internalization of macromolecules (36–38). This uptake pathway is a lipid raft–mediated, clathrin-independent, and caveolae-independent process. However, it is possible that cellular uptake occurs via more than one endocytic pathway depending on, for example, the properties of carrier molecules, concentrations, and the choice of selected cell lines. For example, Fittipaldi et al. (39) concluded that caveolae-mediated endocytosis was the major internalization pathway of the fusion protein for some macromolecular peptides. To discriminate between possible endocytic pathways, we used two types of inhibitors taking into account recent studies, which suggest that a fraction of endocytic uptake of macromolecules, such as oligoarginine peptide and PAMAM dendrimers with a similar structure and molecular weight to 18m-ALA, are inhibited by the macropinocytosis inhibitor EIPA (24). For comparison, the effects of a caveolae-mediated endocytosis inhibitor, NEM, were also examined. We used the PAM 212 keratinocytes in these experiments because they yielded higher PpIX levels; keratinocytes have been reported to be capable of taking up macromolecules by macropinocytosis (40, 41). We found that PpIX levels generated from 18m-ALA were significantly decreased by pretreatment with the macropinocytosis inhibitor EIPA (Figs. 5 and 6), whereas NEM elicited no significant effect. In a control study, neither EIPA nor NEM influenced the production of PpIX in cells after incubation with ALA itself.

We conclude that macropinocytosis has a key role in the uptake mechanism of 18m-ALA dendrimers. These dendrimers contain exposed amino groups that can be protonated at neutral pH causing an overall cationic charge. This study concurs with recent work by Kaplan et al. (36), which showed that macropinocytosis, preceded by ionic interaction between the membrane surface and the macromolecule, was the major endocytic route for cationic poly-arginine–containing macromolecules. The same route should apply to other cationic macromolecules, including poly-lysine drug conjugates (42). Cellular uptake of macromolecular carriers could also occur through various processes, such as phagocytosis for very large carriers or by receptor-mediated endocytosis. However, because there are no specifically targeted ligands for the 18m-ALA dendrimer, it is not expected to be transported by receptor-mediated endocytosis.

In addition to the uptake mechanism, we attempted to assess the rate of internalization of the dendrimer by comparing porphyrin levels induced using short incubation periods from 15 up to 60 min followed by longer periods of incubation in medium without the dendrimer to allow time for porphyrin synthesis. The porphyrin fluorescence levels increased 4-fold from 15- to 60-min exposure for the 4-h measurement time point (Fig. 3A). A comparable rate of increase was observed using ALA itself as shown in the same figure. For the dendrimer, a further factor affecting the porphyrin fluorescence is the rate of ALA cleavage; therefore, we also used much longer measurement time points up to 24 h, which revealed substantial differences in the porphyrin kinetics between ALA and the dendrimer. We found that PpIX production was sustained up to 24 h using the ALA dendrimer, whereas using ALA, significantly less PpIX was present at the 24-h time point (Fig. 3B). Using the dendrimer, the porphyrin levels observed at the 24-h time points were consistently higher compared with the 4-h time points (e.g., using 60-min exposure, 25% higher at 4 h than at 24 h). In contrast using ALA, porphyrin levels were significantly lower at 24 h compared with 4 h, which would be consistent with the metabolism of ALA-induced PpIX formed over the first few hours to haem. These data indicate that ALA is being released gradually from the internalized dendrimer, thereby resulting in sustained porphyrin production in cells up to 24 h after exposure to the compound. Whether all the ALA residues are released over this period is difficult to estimate and this is an area for further study. There are several explanations for such gradual release: (*a*) restrictions in the access of esterases to the ALA residues and (*b*) intracellular esterase levels, bearing in mind the number of ALA residues on each dendrimer (33). Steric hindrance to esterase access would be a rate-determining factor in sequential liberation of the ALA residues, although modifying the linker groups to improve access to esterases should reduce its effect (21). A further possible factor influencing the rate of ALA cleavage is that the dendrimers may be retained within lysosomes because they would become partially protonated at the acidic pH, in which case, the level of lysosomal esterases would become important. We have recently observed lysosomal localization using ALA dendrimers incorporating a fluorescent calcein core[4] in support of this mechanism. Sequestration of macromolecular drugs within endosomal/lysosomal vesicles can be disadvantageous because the bioactive agent coupled to the dendrimer may then be restricted in reaching its target sites. However, this should not apply to ALA dendrimers because it is likely that ALA would be released through lysosomal enzymes.

In previous work (20), we described the synthesis of a dendrimer with a very similar structure also containing 18 ALA residues but with an acetamido linker and presented a feasibility study showing that it was capable of inducing cellular porphyrin synthesis. Although a much smaller range of incubation conditions was used in that study, we showed that this dendrimer was also more efficient than ALA in terms of PpIX production in the PAM 212 cell line: at 0.1 mmol/L using the same assays as in this work, the dendrimer induced approximately seven times more PpIX than ALA after 6-h incubation, which compares with a 10-fold increase compared with ALA for the 18m-ALA dendrimer. Apparently, the use of the propionamido linker in the 18m-ALA may confer a small steric advantage in rendering the ALA residues more accessible to enzymatic cleavage. In our previous studies on smaller dendritic derivatives containing three ALA residues (21), we compared three derivatives with different lipophilicity and showed that higher porphyrin levels could be induced at

[4] Unpublished data.

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low concentrations <0.1 mmol/L in the same PAM 212 cell line for two of the more lipophilic derivatives, together with enhanced phototoxicity, compared with ALA. The least lipophilic 3m-ALA derivative exhibited similar efficacy to ALA itself in two cell lines (21, 22). However, as with 18m-ALA, the smaller dendritic derivatives were less effective at higher concentrations of 1 mmol/L owing we believe to degradation in the culture medium. Because the structures of these smaller derivatives correspond to the 'branches' or dendrons of the larger second-generation ALA dendrimers (i.e., 18m-ALA with six branches/dendrons each bearing three ALA residues), a common degradation mechanism seems likely. The main difference however with the smaller derivatives, which have molecular weights of <1,000, concerns the uptake mechanism. The larger 18m-ALA derivative (molecular weight, 3,678.8) undergoes endocytosis leading to retention of the dendrimer within the cells. The smaller derivatives with their much lower molecular weight seem to be taken up by active transport mechanisms and passive diffusion, with the latter predominating for the more lipophilic derivatives (21, 32).

In summary, the concept of using dendrimers as carriers for the delivery of ALA to tumor cells seems to be a promising method for inducing sustained intracellular porphyrin production for PDT. Further optimization of the dendrimer structure will be needed to confer greater stability to these compounds and comparisons made of cells of different origins (43). Dendrimer-drug conjugates may be used for both topical and systemic administration, and we have shown previously that smaller ALA dendritic derivatives are effective for ALA delivery when applied topically to rat skin or injected in mice (21, 22). The sustained production of PpIX using relatively low concentrations of these dendritic derivatives warrants further systematic study *in vivo*. The use of dendrimers for systemic delivery of ALA over a longer period may have potential applications based on previous work using fractionated ALA dosing (44, 45), where we observed better treatment efficacy and tumor selectivity using fractionated compared with single bolus dosing in a pancreatic tumor model. Moreover, single bolus dosing results in a greater immediate loss of ALA and its metabolite, porphobilinogen, into the urine (46). Many aspects however require further investigation, including the optimum size of the dendrimer and number of ALA derivatives together with their physicochemical properties incorporating the results stemming from the present work.

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