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Aminolevulinic acid dendrimers in photodynamic treatment of cancer and atheromatous disease†

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The use of endogenous protoporphyrin IX after administration of 5-aminolaevulinic acid (ALA) has led to many applications in photodynamic therapy (PDT). We have previously reported that the conjugation of ALA dendrimers enhances porphyrin synthesis. The first aim of this work was to evaluate the ability of ALA dendrimers carrying 6 and 9 ALA residues (6m-ALA and 9m-ALA) to photosensitise cancer cells. For this aim, we employed LM3 mammary carcinoma cells. In these tumour cells, at low concentrations porphyrin synthesis from dendrimers was higher compared to ALA, whereas at high concentrations, porphyrin synthesis was similar from both compounds. Topical application of ALA dendrimers on the skin overlying a subcutaneous LM3 implanted tumour showed no diffusion of the molecules either to distant skin sites or to the adjacent tumour, suggesting a promising use of the ALA macromolecules in superficial cancer models. As a second objective, we proposed the use of ALA-dendrimers in vascular PDT for the treatment of atherosclerosis. Thus, we focused our studies on ALA-dendrimer's selectivity towards macrophages in comparison with endothelial cells. For this aim we employed Raw 264.7 macrophages and HMEC-1 microvasculature cells. Porphyrin synthesis induced in macrophages by 6m-ALA and 9m-ALA (3 h, 0.025 mM) was 6 and 4.6 times higher respectively compared to the endothelial cell line, demonstrating the high affinity of ALA dendrimers for macrophages. On the other hand, ALA employed at low concentrations was slightly selective (1.7-fold) for macrophages. Inhibition studies suggested that ALA dendrimer uptake in macrophages is mainly mediated by caveloae-mediated endocytosis. Our main conclusion is that in addition to being promising molecules in PDT of superficial cancer, ALA dendrimers may also find applications in vascular PDT, since *in vitro* they showed selectivity to the macrophage component of the atheromatous plaque, as compared to the vascular endothelium.

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Introduction

In recent years, (ALA)-mediated photodynamic therapy (PDT) has been an active area of investigation and has become one of the most promising fields in PDT research. ALA is the precursor of the photosensitizer protoporphyrin IX (PpIX). After ALA administration, cells generate PpIX through the haem biosynthetic pathway. The main advantage of PpIX relative to other photosensitizers is the short half-life of its photosensitizing effects which do not last longer than 48 h.[1,2]

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 derivatization or by the use of different delivery vehicles.

Macromolecular drug carriers have been shown to increase cellular uptake of the drugs associated with them by promoting endocytosis. In addition, macromolecules are more easily taken up by tumour cells than normal cells, which is believed to arise from the increased permeability of the tumour vasculature to macromolecules and limited lymphatic drainage.[3,4] After internalisation, lysosomal enzymes hydrolyse any ester linkages to release the free drug. Among the available macromolecules, the dendrimers have the advantages of the high drug payload, and the ability to control and modify the size and lipophilicity of the dendrimer-conjugate for optimising cellular uptake and tissue biodistribution.[5,6]

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Because ALA is a relatively small molecule, it is a suitable candidate for conjugation with macromolecules, such as dendrimers. In principle, dendrimeric nanovehicles bearing a high ALA 'payload' per molecule can deliver a much higher quantity of ALA to cells. In previous work, we have designed a series of novel ALA-containing dendrimers.[7] These compounds contained 3 to 18 ALA residues, and some of them were able to induce PpIX synthesis and cell photodamage in cell cultures. Those studies indicated more efficient and sustained PpIX production than ALA at drug equivalent doses after i.p. administration.[8–10] In addition, the amino groups of the ALA molecules would be protonated *in vivo*, thus conferring good water solubility on the dendrimer. In the present work, we have focused on dendrimers containing 6 and 9 ALA residues for delivery of the pro-drug.

In addition to tumour tissue, atherosclerotic plaque has been proposed as a suitable target for PDT. Drug lipophilicity and high lipid content of vascular plaque were thought to predicate selective uptake.[11] More specifically, the presence of macrophages has been advocated as a reason for the preferential accumulation of PSs in the plaque due to the interaction of PSs with LDL.[12]

Affinity of porphyrins and phthalocyanines for atherosclerotic lesions was reported in the literature several decades ago.[13,14] The development of lasers coupled to fibre optics enabled the possibility of applying PDT to such lesions. PDT of vascular *de novo* atherosclerotic and, potentially, restenotic lesions has been employed for the treatment of hypercholesterolemic rabbits[11,13,15,16] and human peripheral arterial atherosclerosis[17] showing that photoangioplasty holds promise as an alternative intervention for flow-limiting atherosclerosis, either alone or as an adjunct to interventional procedures.[18,19] Endovascular PDT is also being taken under consideration as a method to stabilize the vulnerable plaques by inhibiting inflammation.[20]

In addition to porphyrins, PDT mediated by ALA was employed alone or in combination with angioplasty for endovascular treatment and it proved to be safe and able to reduce restenosis following angioplasty.[21–23]

Since macromolecule carriers promote endocytosis of the carried drug, we propose the use of ALA dendrimers for selective accumulation in the macrophage component of the atheromatous plaques.

The first aim of the present study was to analyse selective accumulation of PpIX formed in tumours from ALA dendrimers employing *in vitro* and *in vivo* models. The second objective was to compare porphyrin synthesis from ALA dendrimers in macrophages and endothelial cells, since they are the most important cell types forming the atheromatous plaques.

Materials and methods

Chemicals

ALA (MW: 168) and MTT were from Sigma Chem Co. 6m-ALA (MW: 1502) and 9m-ALA (MW: 2215) (Fig. 1) were provided by ChemPharm-Research, UK and were freshly prepared in saline for *in vivo* experiments or in water for *in vitro* experiments. The synthesis of the dendrimers was carried out as published previously.[7]

Cell lines

Raw 264.7 macrophages were obtained from ATCC and grown in DMEM (Gibco BRL, Life Technologies Ltd, Paisley, UK) containing high glucose and pyruvate, supplemented with 2 mM L-glutamine, 40 μg gentamycin per ml and 5% fetal bovine serum. Cells were used 48 h after plating.

LM3 cells derived from the murine mammary adenocarcinoma M3[24] were cultured in Eagle's minimum essential medium (Gibco Life Technologies Ltd, Paisley, UK), supplemented with 2 mM L-glutamine, 40 μg gentamycin per ml and 5% fetal bovine serum. Cells were used 48 h after plating.

HMEC-1 human endothelial cells were kindly provided by Dr E.W. Ades (Centers for Disease Control and Prevention, Atlanta, GA). These cells have been immortalised by transfection of human dermal microvascular endothelial cells with a plasmid containing the coding region for the large T antigen of the simian virus 40 A.[25] The cells were grown in endothelial basal medium (EBM-2, Lonza, USA), supplemented with hydrocortisone, 10% serum, hEGF, FBS, VEGF, hFGFB, R3-IGF-1, ascorbic acid, heparin and 40 μg gentamycin from Lonza, USA as well. Cells were used 48 h after plating.

PDT treatment

Cells were incubated in serum-free medium containing ALA or dendrimers and after 3 h, irradiation was performed. After irradiation, medium was replaced by ALA-free medium + serum, the cells were incubated for another 19 h and then tested for viability.

MTT viability assay

Phototoxicity and cell viability were documented by the MTT assay.[26] This is a method based on the activity of mitochondrial dehydrogenases. Following appropriate treatments, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) solution was added to each well at a concentration of 0.5 mg ml^{-1}, and plates were incubated at 37° C for 1 h. The resulting formazan crystals were dissolved by the addition of DMSO and the absorbance was read at 560 nm.

Light source

A bank of 2 fluorescent lamps (Osram L 18W/765) was used. The spectrum of light was between 400 and 700 nm with the highest radiant power at 600 nm. The plates were located at a distance of 14 cm from the light source, and the cells were irradiated from below. The fluence rate was measured with a radiometer (Model 65, Yellow Springs, OH, USA). We used fluences of 0.15 or 0.6 J cm^{-2} and the power density was 0.5 mW cm^{-2}.

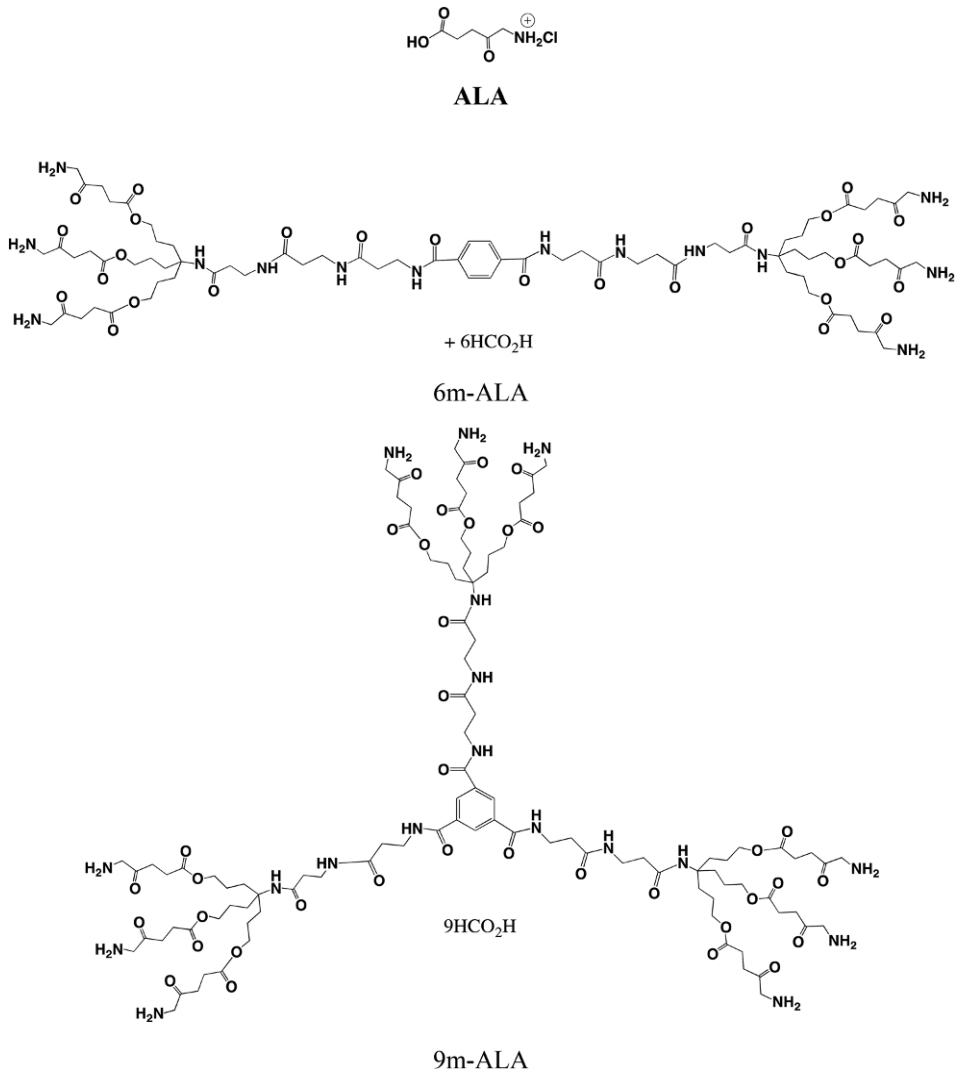

Fig. 1 Structures of ALA and ALA dendrimers.

Endocytosis inhibition

To assess and quantify the endosome–lysosome fusion process after ALA dendrimer uptake, porphyrin synthesis in Raw cells was evaluated after 90 min uptake of 0.3 mM ALA or dendrimers at 18 °C; and after withdrawal of the ALA compounds, the cells were further incubated for 3 h at 37 °C.

To determine the type of endocytosis involved in ALA dendrimer uptake, Raw cells were exposed to the endocytosis inhibitors as follows: 1 min for 0.1 mM *N*-ethylmaleimide (NEM), 1 h for 10 μg ml^{-1} nystatin, 30 min for 10 μg ml^{-1} amiloride and 30 min for 0.5 μg ml^{-1} chlorpromazine. Afterwards, the compounds were withdrawn and the cells were exposed for 3 h to ALA or dendrimers at 0.3 mM and porphyrins were extracted and quantified.

Porphyrin extraction from cells

Porphyrins accumulated within the cells were extracted twice with 5% HCl, leaving the cells standing for half an hour in the presence of the acid at 37 °C. For media determinations, 5% HCl was added and measured directly. These conditions proved to be the optimal for total PpIX extraction. The fluorescence excitation and emission wavelengths were 406 nm and 604 nm, respectively. PpIX (Frontier Scientific, Logan, Utah, USA) was used as a reference standard.

Animals

Male BALB/c mice of 12 weeks old, weighing 20–25 g, were used. They were provided with food (Purina 3, Molinos Río de la Plata) and water *ad libitum*. A suspension of 1.6×10^5cells of the LM3 line was subcutaneously injected into the right flanks of the mice. Experiments were performed at approximately day 20 after the implantation. Tumours of the same uniform size were employed (1 cm diameter). Animals received humane care and protocols were approved by the Argentinean Committee of Animal Ethics (CICUAL, School of Medicine, University

of Buenos Aires) in full accord with the UK Guidelines for the Welfare of Animals in Experimental Neoplasia.[27]

ALA and ALA dendrimer administration

For topical administration, ALA or dendrimers were dissolved in 0.2 ml saline immediately before use. The solutions were applied on the tumour, after shaving the hair and rubbing with a smooth paintbrush for a period of 5 min, a time at which no vestige of lotion was visible. Two areas of skin were investigated: skin over the tumour (SOT), and normal skin taken from the opposite flank, denoted as 'distant skin'.

Fluorescence spectroscopy

In vivo fluorescence measurements were carried out for studying kinetics of PpIX formation after topical application of ALA or ALA peptides. A bifurcated fibre-optic probe was coupled to a Perkin-Elmer LS50B fluorescence spectrometer, and fluorescence from the tissue was detected at the probe tip. Excitation light at a wavelength of 407 nm was coupled into one arm of the bifurcated probe, enabling conduction of excitation to the skin surface and collection of the PpIX fluorescence emission *via* the other arm to the spectrometer. Taking into account the attenuation coefficient for skin, the 407 nm light penetrates deep enough into the skin to excite the PpIX in the epidermis and dermis.[28] The fibre tip was fitted with a rubber spacer that ensured a constant fixed distance of 7 mm between the fibre and the tissue and provided optimal signal collection from the tissue. Fluorescence intensity was measured as a function of time and expressed in arbitrary units at an emission wavelength of 635 nm. In addition, fluorescence emission spectra were recorded to verify that the fluorescence signal corresponded to PpIX.

Porphyrin extraction from tissues

At the time of maximal fluorescence, after ALA or ALA dendrimer application, the animals were sacrificed. Tumour, SOT and distant skin samples were homogenised in a 4 : 1 solution of ethyl acetate :Hglacial acetic acid. The mixtures were centrifuged for 30 min at 3000*g*, and the supernatants were added to an equal volume of 5% HCl. Extraction with HCl was repeated until there was no detectable fluorescence in the organic layer. The aqueous fraction was used for the determination of porphyrins. For fluorometric determination, a Perkin Elmer LS55 spectrofluorometer was used with an emission wavelength of 604 nm and an excitation wavelength of 406 nm, employing PpIX as the reference standard.

Statistical analysis

The values in the figures and tables are expressed as mean ± standard deviations of the mean. A two-tailed Student's *t*-test was used to determine statistical significance between means. *P* values <0.05 are considered significant.

Results

We evaluated porphyrin synthesis from ALA and ALA dendrimers in the mammary carcinoma LM3 cell line (Fig. 2). After a 3 h incubation period, both dendrimers induced higher porphyrin synthesis compared to ALA, employed at low concentrations (54.02 ± 2.3 and 56.7 ± 4.2 ng porphyrins per 10^5 cells for 6m-ALA and 9m-ALA respectively and 15.4 ± 0.8 for ALA) at 0.025 mM concentrations. Plateau values are obtained from ALA at 0.3 mM, whereas from the dendrimers they are obtained at a concentration 10 times lower. The higher ALA payload accounts for the higher rate of porphyrin synthesis, though plateau values obtained are similar to ALA and ALA dendrimers (60 ng porphyrins per 10^5 cells).

By employing longer incubation periods of 24 h the profiles of porphyrin synthesis are similar to the 3 h ones. The amount of tetrapyrroles formed from the dendrimers is still significantly higher than the free drug at low concentrations. However, the differences between porphyrins induced by the dendrimers and by ALA are less marked (53.9 ± 4.2 for ALA; 98.6 ± 9.0 and 101.0 ± 8.2 ng porphyrins per 10^5 cells for 6m-ALA and 9m-ALA respectively) at 0.05 mM concentrations. Plateau values are obtained for both ALA and ALA dendrimers

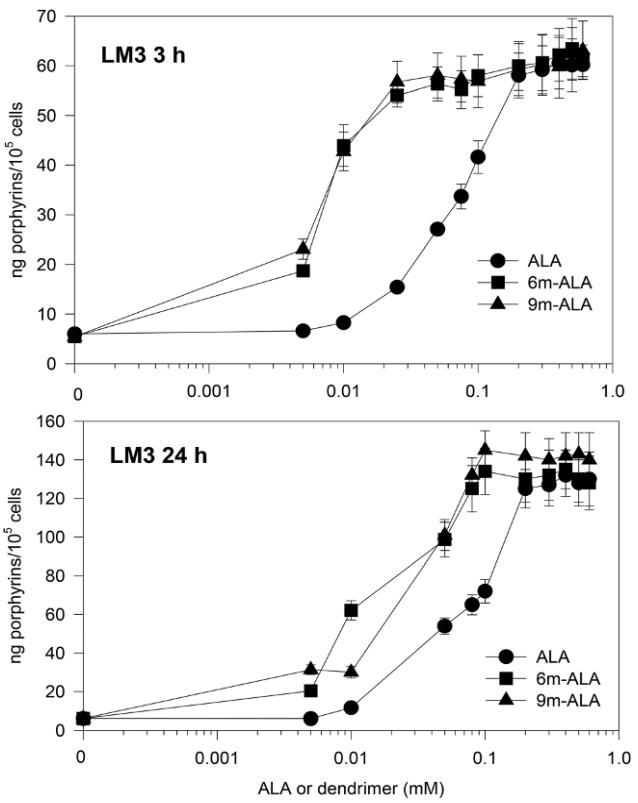

Fig. 2 Porphyrin synthesis from ALA or ALA dendrimers in LM3 cells. Cells were exposed to different concentrations of ALA or ALA dendrimers during 3 h or 24 h. Intracellular porphyrin levels were determined fluorometrically and normalised per number of cells present at the beginning of the experiment.

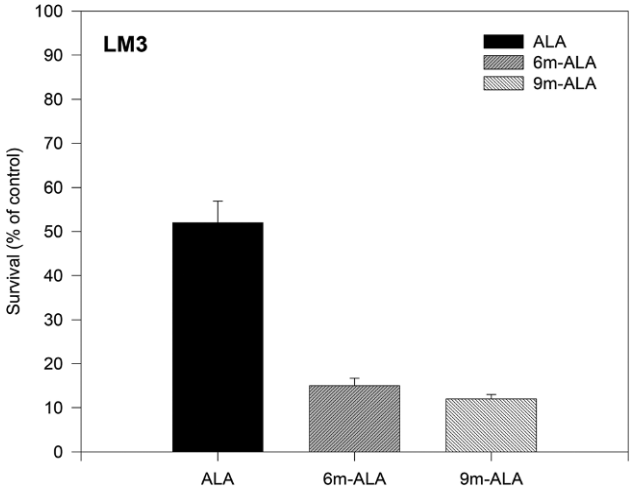

Fig. 3 LM3 cell survival after PDT cells were incubated with 0.025 mM ALA or ALA dendrimers during 3 h. Afterwards, PDT (0.15 J cm^{-2}) was performed, and cell viability was evaluated by the MTT assay, as percentage of control non-irradiated cells.

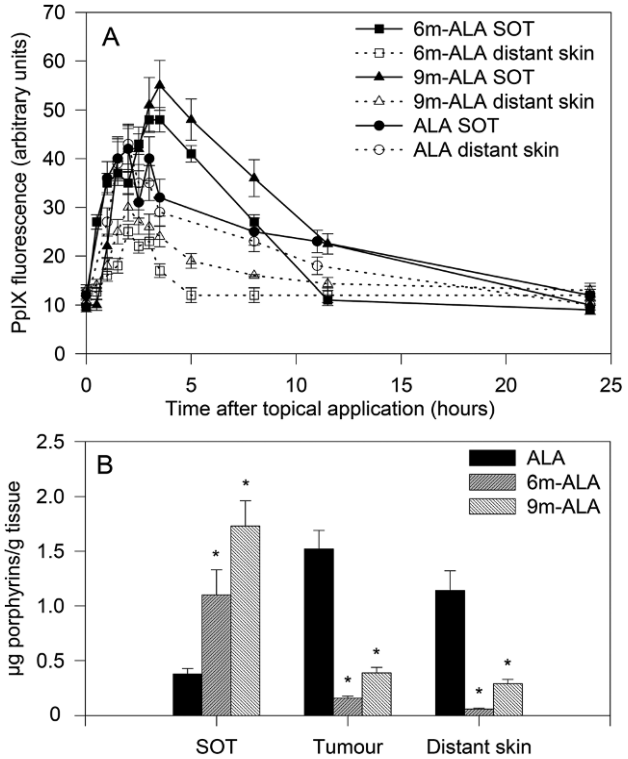

Fig. 4 Porphyrin synthesis in the skin over the tumour and the distant skin after topical application of ALA and ALA dendrimers. ALA and ALA dendrimers were applied topically in equimolar concentrations of 5 mg ALA to the SOT. At different times after application, the porphyrin fluorescence was monitored using a fibre optic probe coupled to a fluorescence spectrometer. Measurements were made on the skin overlying the tumour and over a distant skin area. We herein show the average of three mice per treatment (A). Porphyrin accumulation determined by chemical extraction in tumours, the skin overlying the tumour, and a distant skin site of 3 mice killed at the plateau times obtained from A (B). *$p < 0.05$ as compared to ALA.

at higher concentrations as compared to 3 h incubation, as substrate consumption and maximal PpIX synthesis is around 140 ng porphyrins per 10^5 cells for the three compounds tested.

In line with the patterns of porphyrin synthesis, Fig. 3 shows that the higher the amount of porphyrins, the higher the photocytotoxicity for a given dose comparing ALA with ALA dendrimers. PDT was applied after 3 h incubation with ALA or dendrimers at 0.025 mM, which is a plateau concentration for ALA dendrimers but not for ALA. Under these conditions, ALA induces 50% cellular killing whereas 85 to 90% is obtained employing the dendrimers.

By means of fibre-optic fluorescence detection, we analysed porphyrin formation as a function of time after topical application of ALA and ALA dendrimers on the skin overlying the tumour (SOT) of the implanted mice (Fig. 4A). For comparison of the porphyrin production generated by the dendrimer *versus* ALA, doses of 5 mg of ALA, 7.45 mg of 6m-ALA and 7.32 mg of 9m-ALA were used. These ALA dendrimer doses were used to deliver a drug equivalent dose to ALA, since each dendrimer moiety incorporates 6 or 9 ALA residues respectively.

ALA induces similar profiles of porphyrin production in SOT as compared to distant skin, this being a consequence of rapid diffusion of the molecule to other sites. On the other hand, both dendrimers induce lower amounts of porphyrins in distant skin and higher in SOT as compared to ALA.

Fig. 4B depicts the amount of porphyrins formed 3 h and 4 h after topical application of ALA and ALA dendrimers respectively. Measurements were carried out in tumour, SOT and distant skin after chemical extraction at the plateau times calculated from Fig. 4A. Whereas both tumour and distant skin porphyrins synthesised from ALA dendrimers are much lower compared to ALA, SOT values are significantly higher for the latter, which represents its selective limitation to the area of application, without diffusing to tumour areas.

We also evaluated porphyrin synthesis from ALA and ALA dendrimers in the Raw macrophages 264.7 as well as in the endothelial HMEC-1 cell line (Fig. 5 and 6). After a 3 h incubation period, both dendrimers induced in Raw cells higher porphyrin synthesis compared to ALA, employed at low concentrations (32 ± 3 for ALA; 145 ± 12 and 147 ± 10 ng porphyrins per 10^5 cells for 6m-ALA and 9m-ALA respectively) at 0.025 mM concentrations. Plateau values (around 160 ng porphyrins per 10^5 cells) are obtained from ALA at 0.2 mM, whereas from the dendrimers they are obtained at 0.025 mM, which has an 8 times lower concentration (Fig. 5). After 24 h incubation, overall porphyrin synthesis is significantly increased. At 0.05 mM, ALA induces 68 ± 6 ng porphyrins per 10^5 cells, whereas 146 ± 12 and 280 ± 25 are obtained from 9m-ALA and 6m-ALA respectively.

Similarly, porphyrins induced from low dendrimer concentrations at 3 h are also higher in HMEC-1 cells (Fig. 6) com-

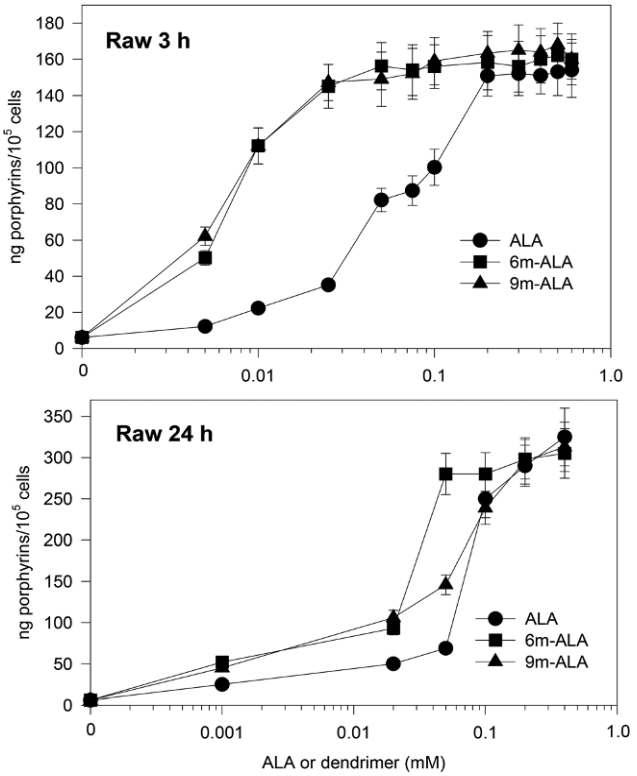

Fig. 5 Porphyrin synthesis from ALA or ALA dendrimers in Raw 264.7 cells. Cells were exposed to different concentrations of ALA or ALA dendrimers during 3 h or 24 h. Intracellular porphyrin levels were determined fluorometrically and normalised per number of cells present at the beginning of the experiment.

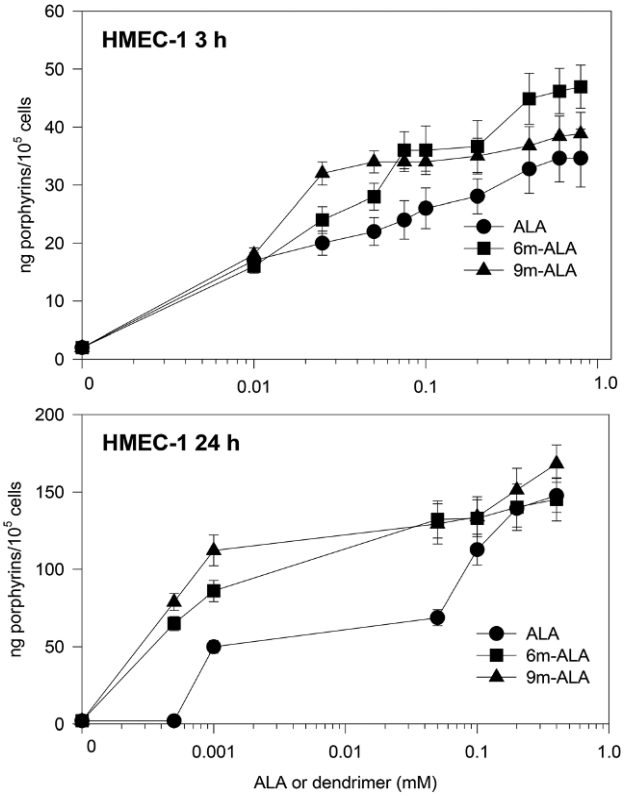

Fig. 6 Porphyrin synthesis from ALA or ALA dendrimers in HMEC-1 cells. Cells were exposed to different concentrations of ALA or ALA dendrimers during 3 h or 24 h. Intracellular porphyrin levels were determined fluorometrically and normalised per number of cells present at the beginning of the experiment.

pared to ALA. Porphyrin values of 20 ± 2 for ALA; 24 ± 2 and 32 ± 2 ng per 10^5 cells for 6m-ALA and 9m-ALA respectively are obtained at 0.025 mM concentrations. Plateau values obtained from 6m-ALA but not from 9m-ALA are significantly higher than those obtained from ALA.

Employing longer incubation times of 24 h, the differences between porphyrins obtained from ALA and those produced by the dendrimers are more marked. Porphyrin values of 68 ± 5 for ALA; 129 ± 12 and 132 ± 13 ng per 10^5 cells for 6m-ALA and 9m-ALA respectively are obtained at 0.05 mM concentrations, and plateau values obtained are 3 times higher than those obtained at the 3 h time point.

In order to assess the selectivity of the dendrimers for the macrophage cells, we calculated the ratio between PpIX synthesised by the macrophages and the endothelial cells at a given concentration (Table 1). The ratio calculated for the 3 h incubation period with ALA is 1.7, whereas the values for the 6m-ALA and 9m-ALA are 6.0 and 4.6 respectively, showing that the ALA dendrimers are largely more selective than the free molecule for the macrophages. Incontrast, at longer incubation time periods, the ratios drop to 2.1 and 1.1 for 6m-ALA and 9m-ALA respectively, whereas 1 is the ratio obtained for ALA.

Employing 3 h incubation period in the presence of 0.025 mM of ALA or dendrimers, PDT induces mild conditions of cell death for HMEC-1 cells, whereas macrophages treated with ALA dendrimers but not with ALA are completely killed (Fig. 7).

Table 1 Porphyrin synthesis ratios between macrophages and endothelial cells exposed to ALA or dendrimers[a]

	ALA	6m-ALA	9m-ALA
Macrophages/endothelial cells at 3 h (0.025 mM)	1.7	6.0	4.6
Macrophages/endothelial cells at 24 h (0.05 mM)	1.0	2.1	1.1

[a] The ratios of porphyrin synthesis are calculated on the basis of the values of 0.025 and 0.05 mM for time incubation periods of 3 and 24 h respectively taken from Fig. 5 and 6.

Since the ALA payload of the dendrimers is much higher, we calculated the amount of ALA delivered by the dendrimers expressed in molar equivalents of ALA (Table 2), and we listed a set of porphyrin synthesis values taken from Fig. 2, 5 and 6 in order to establish an accurate comparison. It appears that at low 6m-ALA concentrations (lower than plateau values), the amount of porphyrins synthesised by LM3 and Raw cells is

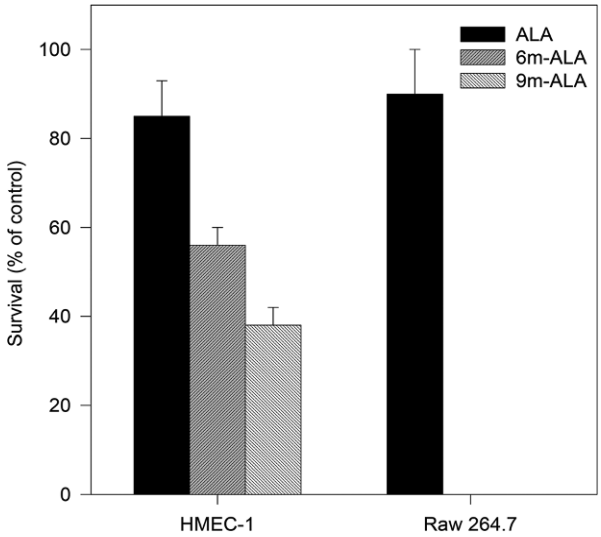

Fig. 7 HMEC-1 and Raw264.7 cell survival after PDT. Cells were incubated with 0.025 mM ALA or ALA dendrimers during 3 h. Afterwards, PDT (0.6 J cm^{-2}) was performed, and cell viability was evaluated by the MTT assay, as the percentage of control non-irradiated cells.

Table 2 ALA equivalent dose delivered by the dendrimers (amount of ALA, as expressed in the ALA molar equivalent value) according to the prodrug concentration and porphyrin synthesis in the different cell lines at 3 h (expressed as ng porphyrins per 10^5 cells)

Prodrugs	Prodrugs (mM)	ALA molar equivalent (mM)	LM3	Raw	HMEC-1
ALA	0.005	0.005	6.6	12.2	2.0
	0.01	0.01	8.2	22.3	17.0
	0.05	0.05	27.1	82.1	22.3
	0.1	0.1	41.6	100	26.1
	0.5	0.5	60.1	153	32.7
6m-ALA	0.005	0.03	18.7	50.2	5.3
	0.01	0.06	43.9	112	16.2
	0.05	0.3	56.3	156	28.5
	0.1	0.6	58.0	156	36.2
	0.5	3	63.4	162	44.9
9m-ALA	0.005	0.045	23.1	62.1	6.7
	0.01	0.09	42.5	112	18.4
	0.05	0.45	58.1	149	34.2
	0.1	0.9	56.8	159	34.3
	0.5	4.5	62.4	168	36.8

higher than that obtained from ALA, whereas 9m-ALA induces similar porphyrin values as compared to ALA, even at low concentrations. In contrast, ALA dendrimers seem to be less efficient than ALA in HMEC-1 cells, throughout the concentration range assayed.

To gain insight into the mechanisms of endocytosis, Raw cells were pre-treated with drugs that selectively inhibit clathrin-dependent endocytosis (chlorpromazine), caveolae-dependent endocytosis (nystatin and NEM), and macropinocytosis (amiloride) before being exposed to the ALA dendrimers (Table 3). Possible drug-induced cytotoxic effects were assessed by MTT cell viability assays and observation of cellular morphological changes. Minimal cellular cytotoxicity was observed in drug-treated cells throughout the spectra of concentrations used in these experiments.

Table 3 Percentage of endocytosis inhibition in Raw 264.7 macrophages[a]

	ALA	6m-ALA	9m-ALA
N-Ethylmaleimide	10 ± 1.1	46 ± 6.2	43.7 ± 5.3
Nystatin	8.7 ± 0.9	40 ± 3.8	35.9 ± 3.4
Amiloride	7.1 ± 0.5	7.9 ± 0.9	3.9 ± 5.1
Chlorpromazine	2.9 ± 0.4	12.7 ± 1.1	11.7 ± 1.8
18 °C	8.3 ± 0.7	48.3 ± 5.3	38.3 ± 4.2

[a] Raw 264.7 cells were preincubated with the endocytosis inhibitors and subsequently incubated with 0.3 mM ALA or dendrimers. After 3 h of incubation, porphyrin synthesis was evaluated. Alternatively, the macrophages were incubated for 3 h at 18 °C in the presence of ALA or dendrimers, which were withdrawn and afterwards porphyrin synthesis was evaluated. The percentage of endocytosis inhibition was inferred from the decrease of porphyrin synthesis.

Employing caveolae-mediated endocytosis inhibitors, porphyrin synthesis from both dendrimers was impaired 35–45%, thus showing that this process is involved in the uptake of the dendrimers. On the other hand, neither macropinocytosis nor clathrin-mediated endocytosis was related to the process.

It has been previously demonstrated that the transport between endosomes and lysosomes is blocked at approximately 18 °C.[29,30] Accordingly, whether this incubation temperature could block the transport of dendrimers from endosomes to lysosomes in the Raw macrophages was evaluated. We observed a 48% and 38% inhibition of porphyrin synthesis for 6m-ALA and 9m-ALA respectively by blocking endosome–lysosome fusion.

Discussion

Improved targeting to solid tumours can be achieved by conjugation of anticancer drugs to macromolecules such as dendrimers.[31] In the present work we found that ALA dendrimers are promising in the treatment of superficial cancers since they induce high amounts of PpIX in the area of application with minimal diffusion to distant tissues. The ratios between SOT and distant normal skin porphyrins are 1.1 for ALA, 2.1 for 6m-ALA and 2.5 for 9m-ALA, thus showing a marked confinement to the site of application of the dendrimers, though the diffusion from the SOT to the adjacent tumour is minimal.

The compound 6m-ALA with shorter branches than the current compound has previously been instilled into rat bladder tumours,[32] and it was found that fluorescence ratios between tumour to normal urothelium were around 1.2, and tumour to muscle were 2.0, which is very similar to the ratios obtained employing ALA hexyl ester. In addition, 6m-ALA showed higher penetration depths and absence of systemic reabsorption.

After topical administration of the dendrimers, the time of maximum porphyrin levels appeared to be delayed compared to ALA application by about 1 h. We have found a similar behaviour for the 3m-ALA dendrimer,[8] and we have ascribed it to the retention of the more lipophilic dendron within the stratum corneum at the application site.

The role of the stratum corneum retention appears to be crucial in the use of ALA esters and ALA dendrimers. For 6m-ALA and 9m-ALA we found that skin sites distant from the site of topical application exhibited significantly lower porphyrin levels than ALA. We have previously found this pattern for the 3m-ALA dendron,[8] as well as for the hexyl ester derivative of ALA.[33] Slow hydrolysis of ALA from dendrimers has been previously reported *in vivo* for 6m-ALA in tumour cells.[32] In contrast, gradual release of the ALA residue from the dendrimer has not been observed for topical application of dendrimers.[8]

We have previously found that PpIX levels generated from the dendrimer-containing 18 ALAs, 18m-ALA, were significantly decreased by pre-treatment with the macropinocytosis inhibitor 5-(*N*-ethyl-*N*-isopropyl)amiloride, whereas NEM elicited no significant effect.[9] However, the entry and subsequent trafficking of the nanoparticles can both depend on the cell type and the particle charge and size.[34] In the present work we found that caveolae-mediated endocytosis is involved in 6m-ALA and 9m-ALA uptake, whereas neither macropinocytosis nor clathrin-mediated endocytosis is associated with the ALA dendrimer's entry into the macrophages. However, this is not surprising, since it has previously been reported for PAMAM dendrimers that the entry mechanism appeared to depend on the dendrimer generation, with the smaller dendrimers taken up by clathrin-dependent endocytosis and the bigger ones by multiple routes.[35,36]

There are several different types of endocytosis, all based on formation of intracellular vesicles following invagination of the plasma membrane or ruffling giving rise to larger vesicles. These endocytic pathways are distinguished by specific molecular regulators.[37] Two main pathways have been identified for receptor-mediated endocytosis: the clathrin-dependent and the caveolae/lipid raft-dependent endocytic pathways. Clathrin-dependent endocytosis is the most well characterized mechanism for mediating the internalization of membrane receptors into cells. It is also important for intracellular trafficking at the *trans*-Golgi network and endosomes. Caveolae/raft-dependent endocytosis is involved in multiple biological processes, including virus entry into host cells, internalising glycophosphatidyl inositol-anchored proteins and regulating certain signalling cascades.[38] Macropinocytosis is a special case of clathrin-, caveolae- and dynamin-independent endocytosis, which is initiated by transient activation of receptor tyrosine kinases by growth factors. The receptor activation mediates a signalling cascade that leads to changes in the actin cytoskeleton and triggers the formation of membrane ruffles. These membrane ruffles protrude to engulf the surrounding fluid and nutrients in the extracellular milieu.[39] All of these endocytic pathways have been shown to be involved in the uptake of nanomedicines.[37]

Cell-specific differences also play a role in the uptake of macromolecules: different kinds of dendrimers have been taken up by clathrin-mediated endocytosis in Caco-2 cells[35] and by macropinocytosis in A549 cells.[40,41] In addition, gene delivery by dendrimers has been also shown to use different internalisation pathways in different cells, but that caveolae form a preferential route.[42,43]

In addition, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and macropinocytosis processes have been shown to be active in Raw 264.7 macrophages, and involved in the uptake of micellar nanoparticles independently of the molecules charge.[44] Similarly, there are several main endocytic pathways in endothelial cells, including clathrin- and caveolae-mediated endocytosis, phagocytosis and macropinocytosis. The latter may be of interest for intracellular drug delivery to endothelial cells involved in inflammation.[45,46] However, macrophages incorporate ALA dendrimers at rates of 6 or 4.6 times higher than HMEC-1 endothelial cells, thus showing that the higher endocytic ability of the macrophages accounts for ALA dendrimers selectivity.

Even though, caveolae are present in many cell types they are especially abundant in endothelial cells. Another major difference is that the caveolar uptake is a non-acid and has no digestive route of internalization. In addition, evidence for caveolin expression in macrophages has been scarce and conflicting,[47] however caveolin-1 mRNA was detected in Raw 264.7 macrophages.[48] Caveolae do not suffer a drop in pH, and most molecules that are internalised by caveolae can be directly transported to the Golgi and/or endoplasmic reticulum, thus avoiding normal lysosomal degradation.[49] This implies that acidic esterases would not be involved at all in the release of ALA residues from the dendrimers.

The capability to selectively kill macrophages has applications in treating cancer and in the detection and therapy of vulnerable atherosclerotic plaque and possibly for autoimmune disease and some infections.[50] The efficacy of vascular PDT should be based on the selective accumulation of photosensitizer by atheromatous plaque and optimum drug-light interval. Differential kinetics of PpIX formation in the different cell types of a plaque was demonstrated by Jenkins *et al.*[51] After ALA administration to normal pigs, PpIX fluorescence peaked in the adventitia, intima and medial layers at 1.5, 4 and 6 h respectively. At 6 h after ALA administration the ratio of media (muscle cells) : intima (endothelium) PpIX was about 2.

In the present work, *in vitro* studies employing Raw 264.7 macrophages and HMEC-1 endothelial cells suggested that ratios of porphyrin synthesis of 1.7 for macrophage–endothelial cells from ALA are improved. The ratios obtained at 3 h and 0.025 mM concentrations are 6.0 and 4.6 for 6m-ALA and 9m-ALA respectively, suggesting that the dendrimers are better tools than ALA to selectively destroy atheromatous plaques by means of vascular PDT.

In the process of PpIX synthesis from ALA dendrimers, several steps are involved: (a) dendrimer entry into the cells, (b) enzymatic hydrolysis and release of ALA, (c) PpIX synthesis

by six enzymes of the haem biosynthetic pathway. We calculated the amount of ALA delivered from the dendrimers expressed in molar equivalents of ALA, and we found that in both tumour and macrophage cells the dendrimers are more efficient than ALA at low concentrations. This has been previously found in other tumour cells for ALA esters and dendrons,[8,33] and is highly influenced by the importance of the pro-drug entry into the cells.

At longer incubation time periods, the differences between the drug and the prodrugs become minimal, and this may be due to a major and limiting role of the PpIX synthesis step rather than the ALA or pro-ALA entry step. In previous work we have found that at high (plateau) concentrations, the regulation of ALA conversion into porphyrins is driven by the enzyme porphobilinogenase.[52]

On the other hand, for HMEC-1 cells the dendrimers do not enhance PpIX production from ALA even at low concentrations, and we ascribe this to their low endocytic ability.

Considering the different ALA payloads of both dendrimers and the similarity of the porphyrin synthesis curves as a function of the prodrug concentration for the three cell lines and both time points analysed, we conclude that hydrolysis from 9m-ALA is not complete, and that 6m-ALA is an equal or better PpIX producer than the 9 ALA containing dendrimer.

In animal models, it was shown that after ALA administration, the fluorescence intensity of PpIX in the atheromatous plaque reached the peak 2 h after injection and was around 10 to 12 times higher than that of adjacent normal vessel segments, having a positive correlation with the macrophage content.[53,54] In addition, Peng *et al.*[53] demonstrated that the ALA-derived PpIX can be employed to reflect the macrophage content in the plaque, and that ALA-PDT could reduce the macrophage content.

In addition to its potential use for PDT of cancer, the key conclusion of this work is that ALA dendrimer mediated photoangioplasty is a promising alternative for the treatment of cardiovascular obstructive diseases. Animal model studies employing ALA dendrimers are needed to further assess the selectivity of PpIX from dendrimers in atheromatous plaques, as well as further extend our studies to vascular smooth muscle cells.

Abbreviations

ALA	5-Aminolevulinic acid
MTT	(3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide)
PDT	Photodynamic therapy
PpIX	Protoporphyrin IX

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