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Sustained and efficient porphyrin generation *in vivo* using dendrimer conjugates of 5-ALA for photodynamic therapy

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ABSTRACT

The use of endogenous protoporphyrin IX (PpIX) after administration of 5-aminolaevulinic acid (ALA) has led to many applications in photodynamic therapy (PDT). However the efficacy of ALA-PDT is sub-optimal for thicker tumours and improved ALA delivery and therapeutic response are required. We have investigated the conjugation of ALA to a second-generation dxcendrimer for enhancing porphyrin synthesis *in vitro* and *in vivo* in a murine tumour model using systemic i.p. administration. *In vitro*, the dendrimer was more efficient than ALA for porphyrin synthesis at low concentrations in good correlation with higher cellular ALA dendrimer accumulation. *In vivo*, the porphyrin kinetics from ALA exhibited an early peak between 3 and 4 h in most tissues, whereas the dendrimer induced sustained porphyrin production for over 24 h and basal values were not reached until 48 h after administration. Integrated porphyrin accumulation from the dendrimer and ALA, at equivalent molar ratios, was comparable showing that the majority of ALA residues were liberated from the dendrimer. The porphyrin kinetics appear to be governed by the rate of enzymatic cleavage of ALA from the dendrimer, which is consistent with *in vitro* results. ALA dendrimers may be useful for metronomic PDT, and multiple low-dose ALA-PDT treatments.

1. Introduction

Photodynamic therapy (PDT) of cancer is based on the administration of a photosensitising compound and subsequent irradiation with light of an appropriate wavelength leading to damage of the treated tissue [1]. The use of endogenous protoporphyrin IX (PpIX) generated through the haem biosynthetic pathway after administration of 5-aminolaevulinic acid (ALA) has led to many applications in PDT, particularly for treatment of cutaneous basal call carcinoma [2,3]. Clinical advantages of this approach include the choice of either topical or systemic administration of ALA, and short-lived cutaneous photosensitivity together with selective epithelial photosensitisation rendering it suitable for treatment of early tumours [4]. ALA also has great potential as a photodiagnostic or photodetection agent in clinical practice. The use of ALA-induced PpIX fluorescence is currently being exploited for diagnosis of bladder cancer, intraepithelial lesions of the cervix and lung cancer and fluorescence-guided surgery for resection of malignant gliomas [5]. In the latter case, ALA is systemically administered using an oral dose of 20 mg/kg which is well tolerated by patients. However higher oral doses up to 60 mg/kg, used for example for PDT of dysplasia in Barrett's Oesophagus [4], induce adverse side-effects including hypotension and vomiting, which can however be reduced by the use of fractionated ALA dosing.

The efficacy of ALA-PDT is however limited by the hydrophilic nature of the molecule, leading to poor penetration through tumour. Several chemical modifications have been made both on the N- and C-terminii of ALA to induce higher PpIX production and photosensitisation [6,7]. Esterification of ALA with aliphatic lineal and cyclic alcohols was found to reduce the amount of ALA required for cell killing [8–11]. In addition, ALA-peptide prodrugs based on phenylalanyl-ALA conjugates enhanced by a factor of 5 the fluorescence induced by ALA *in vitro* [12]. Another approach for enhancing ALA-PDT is the combination of ALA with other agents, including chemotherapy agents [13] and iron chelating agents to inhibit ferrochelatase-catalysed conversion of PpIX to haem [14,15].

There is currently considerable interest in the development of new drug delivery systems using nanoparticles based on micelles and

Abbreviations: ALA, 5-aminolaevulinic acid; 18m-ALA, 1,3,5-Tris[*N*-(*N*-bis{*N*-[tris(5-aminolaevulinyloxymethyl)methyl]propionamido}propionamido)carbamido]benzene 18·Trifluoroacetic acid; PpIX, Protoporphyrin IX; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide; PDT, photodynamic therapy; TCA, Trichloroacetic acid; TFA, Trifluoroacetic acid.

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liposomes or polymeric nanocarriers, and several studies have demonstrated improvements in the efficacy of anti-cancer agents using this approach [16,17]. Moreover, improved targeting to solid tumours can also be achieved upon conjugation of anticancer drugs to nanoparticles [18], either through active or passive targeting [19,20]. Recent advances in polymer chemistry now enable the synthesis of structurally-defined, hyperbranched polymers known as dendrimers, which can be conjugated with drug molecules [21–23]. The advantages of using dendrimers are 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 [21,24]. Accordingly, the behaviour of the conjugated drug is modulated by the properties of the dendrimer nanovehicle for optimising the drug delivery.

In previous work we reported the synthesis of a series of novel ALA-containing dendrimers, which were shown to induce porphyrin formation in cells [25]. The interior or core of the dendrimers was hydrophobic whilst the exterior, or periphery was relatively hydrophilic. These compounds contained from 6 to 18 ALA residues. Three first-generation dendrimers bearing either 6 or 9 ALA residues were attached to an aromatic or aliphatic core and two second-generation 18-ALA containing dendrimers were also synthesised with an additional spacer unit between the ALA residues and the core.

More recently we have compared the properties of smaller ALA dendritic derivatives in the PAM 212 keratinocyte cell line. We studied three different derivatives (G0 dendrons) which all incorporated three ALA molecules conjugated as esters via methyl or propyl linkers to a central tertiary carbon whose remaining terminal bore an amino or aminobenzyloxycarbonyl or nitro group respectively. The three compounds were more efficient at low concentrations compared to equimolar ALA for porphyrin production, with the most efficient incorporating the longer propyl linker, suggesting that lipophilicity and steric hindrance were the factors that mostly governed PpIX synthesis [26]. We further evaluated the efficacy of the dendron, aminomethane tris-methyl 5-aminolaevulinic acid, *in vivo* and *in vitro* [27]. Although the dendron was taken up with comparable efficiency to ALA, there was only partial intracellular liberation of ALA residues. Both systemic and topical administration of the dendron to tumour-bearing mice induced higher porphyrin levels than the widely investigated hexyl ester derivative in most tissues studied, although it was not possible to surpass the levels induced by ALA.

In this study we have investigated a second-generation dendrimer bearing eighteen ALA residues (18m-ALA), which we have previously studied in the PAM 212 keratinocyte and A431 human epidermoid carcinoma cell lines [28]. In that work we demonstrated efficient porphyrin sensitisation and cell kill following light exposure using the dendrimer. The ALA residues are coupled to the dendrimer by ester linkages so that ALA can be released within the cells for subsequent metabolism to protoporphyrin IX. The aim of the present work was primarily to evaluate *in vivo* the efficacy of the 18m-ALA dendrimer for inducing PpIX synthesis, and carry out further *in vitro* evaluation.

2. Materials and methods

2.1. Cell line and cell culture

Cell line LM3 [29] derived from the murine mammary adenocarcinoma M3 was cultured in minimum essential Eagle's medium (Gibco Life Technologies Ltd, Paisley, UK), supplemented with 2 mM L-glutamine, 40 μg gentamycin/ml and 5% fetal bovine serum, and incubated at 37 °C in an atmosphere containing 5% CO_2. Cells were used 48 h after plating.

2.2. Chemicals

ALA was purchased from Sigma-Aldrich (Poole, UK). The structures of ALA and the dendrimer containing 18 ALA residues coupled via ester linkages (MWt: 3679, abbreviated herein to 18m-ALA) are shown in Fig. 1. The compound was prepared as a trifluoroacetic acid salt (TFA). The synthesis of the dendrimer was carried out using the convergent method, as described previously [25]:1,3,5-Tris[*N*-(*N*-bis{*N*-[tris(5-aminolaevulinyloxymethyl)methyl]propionamido}propionamido)carbamido]benzene 18·Trifluoroacetic Acid (FWt: 5731). The convergent growth strategy adopted for the synthesis of 18m-ALA dendrimer enabled the dendrimer to be assembled in segments with a finite and constant number of reactive sites. The building blocks, (dendrons) containing three ALA molecules with protective amino group were constructed first of all, giving a small number of possible side-reactions (or 'missing' reactions) per step. Each synthesised generation of dendrons was purified and characterized fully before proceeding to the next step, by HPLC, NMR, Mass spectroscopy, and the purity reached 99% in most of the steps. These dendrons were attached to a multipodent core unit in the final steps of the dendrimer construction. Although purification of the more bulky higher-generation dendrons is more complicated, with careful purification after each step, we have managed to synthesise the 18m-ALA dendrimer with approximately 97% purity according to HPLC, NMR, and CHN elemental analysis. Also, the use of trifluoroacetic acid (TFA), which is very mild reagent for cleavage of the BOC protective amino group of ALA in the final step, guaranteed that the dendrimer remained intact [25].

2.3. Preparation of solutions

18m-ALA and ALA were dissolved in water just before use. Addition of ALA and 18m-ALA at non-toxic concentrations did not change the pH of the cell medium.

2.4. 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 excitation and emission wavelengths of light used producing the highest fluorescence were 406 nm and 604 nm, respectively. PpIX (Frontier Scientific, Logan, Utah, USA) was used as a reference standard.

2.5. MTT viability assay

Phototoxicity and cell viability was documented by the MTT assay based on the activity of mitochondrial dehydrogenases. Following treatment, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazoliumbromide) purchased from Sigma-Aldrich (Poole, UK) solution was added to each well in a concentration of 0.5 mg/ml, and plates were incubated at 37 °C for 1 h. The resulting formazan crystals were dissolved by the addition of DMSO and absorbance was read at 560 nm.

2.6. ALA and PBG determinations in cells

Cells were seeded in 100 mm dishes. After 72 h, the medium was removed and the cells were exposed 3 h to 0.1 mM ALA or 18m-ALA in medium without serum. Afterwards, cells were washed 4 times with PBS and 5% TCA was added. After scrapping, the cells were centrifuged and the supernatant was employed for ALA and PBG determination using a modification of the method developed by Mauzerall and Granick [30]. Briefly, for ALA determination, a condensation reaction was carried out in the presence of acetyl acetone, and after centrifugation the resulting pyrroles were quantified by addition of the Ehrlich reactive measuring absorbance at 555 nm. Standards of ALA and 18m-ALA were also condensed and employed for calculations. For PBG determination, the Ehrlich reagent was added to the deproteinised TCA supernatant. ALA values were obtained by

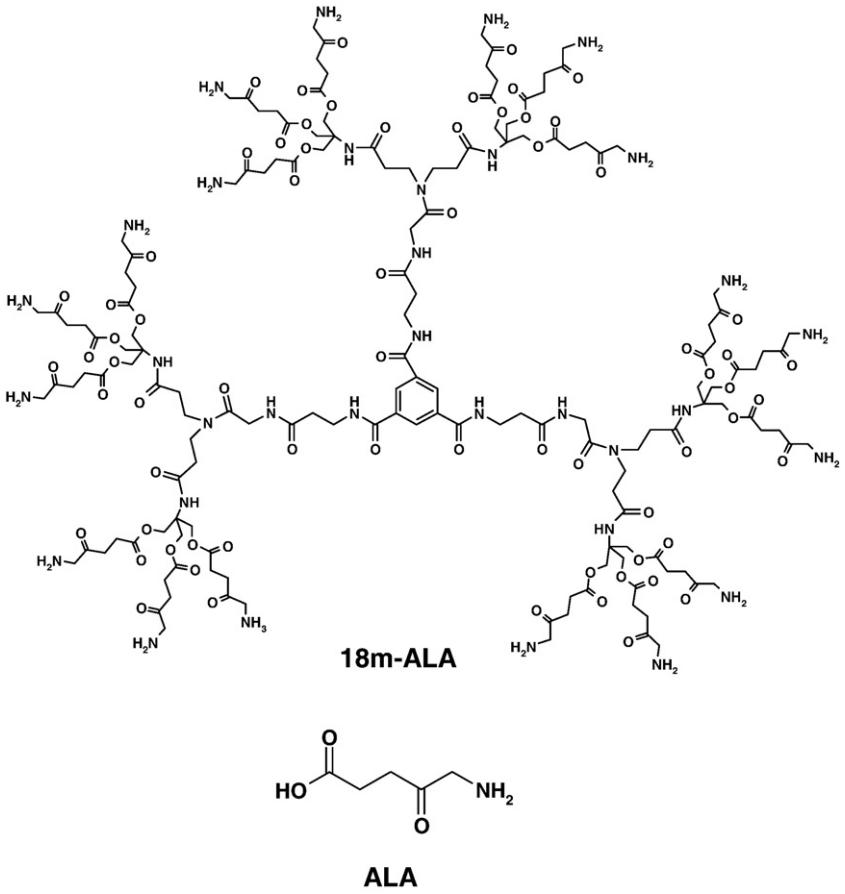

Fig. 1. Structures of 18m-ALA and ALA.

subtracting PBG values from the total condensed pyrroles. Prior to these experiments, we found that only one ALA residue of the 18m-ALA is condensed with the acetylacetone to form a coloured pyrrole. Accordingly, equimolar concentrations of ALA and 18m-ALA should give identical absorbances at 555 nm. The reason for the reaction of just one ALA per dendrimer molecule may be the interaction of the other formed pyrroles leading to the formation of colourless dipyrroles [30]. In addition, the reaction product gives identical absorption spectra for ALA and 18m-ALA.

2.7. ALA and 18m-ALA separation using ion-exchange column chromatography

In previous experiments we established that ALA can be separated from dendritic ALA derivatives by our method designed to separate ALA esters from ALA [30]. Cells were seeded in 100 mm dishes. A pool of two dishes per point was used. After 72 h, medium was removed and cells were exposed 3 h to 0.1 mM ALA or 18m-ALA in medium without serum. Afterwards, cells were washed 4 times with PBS and 5% TCA was added. After scrapping, the cells were centrifuged and the two dishes were pooled. An aliquot of the supernatant was employed for ALA and PBG determinations as described above. The rest of the supernatants were passed through a column of Dowex 50×8 resin (Sigma-Aldrich, Poole, UK)as described elsewhere [31]. ALA was separated from 18m-ALA, and the percentage of the total was calculated after subtracting the PBG contribution.

2.8. Animals

Male BALB/c mice 12 weeks old, weighing 20–25 g were used. They were provided with food (Purina 3, Molinos Río de la Plata, Argentina) and water *ad libitum*. A suspension of 1.65×10^5 cells of the LM3 cell line was subcutaneously injected on the flanks of male BALB/c mice. Experiments were performed at approximately day 20 after implantation. Tumours of the same uniform size were employed (1 cm diameter). Animals received human care and were treated in accordance with guidelines established by the Animal Care and Use Committee of the Argentine Association of Specialists in Laboratory Animals (AADEALC), in full accord with the UK Guidelines for the Welfare of animals in Experimental Neoplasia [32]. The protocol has received approval from the local Committee of the School of Sciences, University of Buenos Aires.

2.9. ALA and 18m-ALA administration

ALA (HCl salt) and 18m-ALA (TFA salt) were dissolved in saline in a final volume of 0.15 ml immediately before intraperitoneal (i.p.) injection. For comparison of the porphyrin production generated by the dendrimer versus ALA, doses of 14 mg of 18m-ALA and 7.4 mg ALA respectively were used. This ALA dose was used to deliver a drug equivalent dose to the 18m-ALA dose, since each dendrimer moiety incorporates eighteen ALA residues. The ALA dose corresponds to 300 mg/kg for a 25 g mouse.

2.10. Tumour and normal tissue porphyrin extraction

After ALA or 18m-ALA injections, animals were sacrificed. Before killing, mice were injected with heparin (0.15 ml, 1000 UI) and after sacrifice, they were perfused with 200 ml of sterile saline. The tumour samples were homogenised in a 4:1 solution of ethyl acetate: glacial acetic acid. The mixtures were centrifuged for 30 min at 3000 g, and the supernatants were added with an equal volume of 5% HCl.

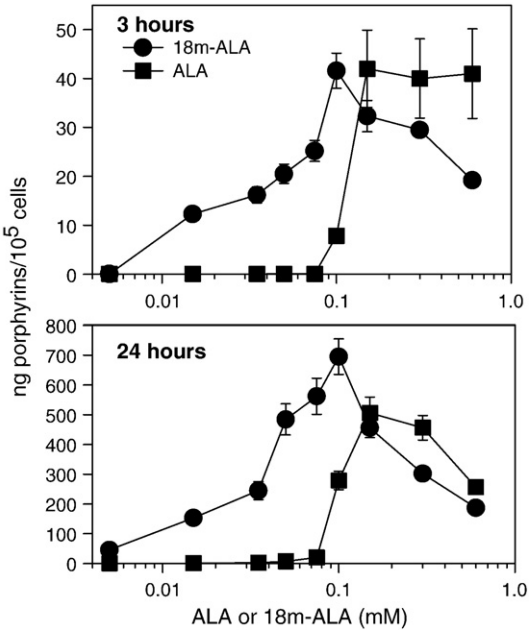

Fig. 2. Porphyrin synthesis from ALA or 18m-ALA in LM3 cells. Cells were exposed to ALA or 18m-ALA during 3 or 24 h. Porphyrins were chemically extracted and quantified fluorimetrically.

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 fluorimetric determination, a Shimadzu RF-510 spectrofluorimeter was used (Shimadzu Corp. Kyoto, Japan), with an emission wavelength of 604 nm and an excitation wavelength of 406 nm, employing PpIX reference standard. Data points for control animals with no drug administered are shown in the figures below without subtraction of the basal levels.

2.11. Statistical analysis

The unpaired *t*-test was used to establish the significance of differences between groups. Differences were considered statistically significant when $P<0.05$. *In vitro* experiments: three independent experiments, in triplicates. *In vivo* experiments: Three mice per group were employed.

3. Results

Fig. 2 shows that LM3 adenocarcinoma cells, which were used in subsequent tumour model experiments, exhibited greater porphyrin synthesis from 18m-ALA at low concentrations (both at 3 and 24 h) upon exposure to equimolar concentrations of ALA and 18m-ALA, ie 18 times higher than the ALA equivalent dose. Porphyrin synthesis was much higher at 24 h for both compounds. However, at concentrations

Table 1
ALA and PBG accumulation in cells after exposure to ALA or 18m-ALA.

	pmol of ALA of ALA + 18m-ALA/10^5 cells	pmol of PBG/10^5 cells	% of hydrolysed ALA
Control	1.62 ± 0.07	0.75 ± 0.03	100%
ALA (3 h)	13.0 ± 0.9	1.52 ± 0.11	100%
18m-ALA (3 h)	97.9 ± 6.2	2.71 ± 1.30	25.6%
ALA (24 h)	14.8 ± 1.6	5.0 ± 0.82	100%
18m-ALA (24 h)	161.3 ± 9.8	18.5 ± 2.2	40.3%

ALA and PBG were evaluated after 3 h or 24 h exposure to 0.1 mM ALA or 18m-ALA. ALA or 18m-ALA intracellular uptake was determined by the addition of the Ehrlich reagent according to Materials and methods. PBG values were determined by the same method without condensation. The percentage of hydrolysed ALA was determined after ionic exchange chromatography separation.

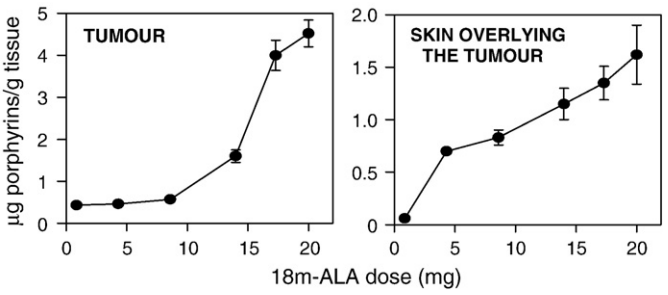

Fig. 3. Porphyrin accumulation in LM3 tumour and skin overlying the tumour after i.p. administration of increasing 18m-ALA doses. Different amounts of 18m-ALA were injected i.p. to mice. Twenty four hours later, tissues were excised and porphyrins extracted. Each data point represents the average of three determinations. Error bars show standard deviations.

higher than 0.1 mM, a decrease in porphyrin synthesis was found particularly at the longer 24 h incubation time. The reduction in efficacy at the longer incubation times appears to be due to toxicity according to the MTT viability assay (data not shown). In our previous study [28] we also observed some precipitation at higher concentrations which was proposed to result from condensation reactions between ALA dendrimer residues resulting in polymeric degradation products.

The intracellular ALA content was also assessed. Table 1 shows that accumulation of ALA/18m-ALA at 0.1 mM was 7.5 times higher from

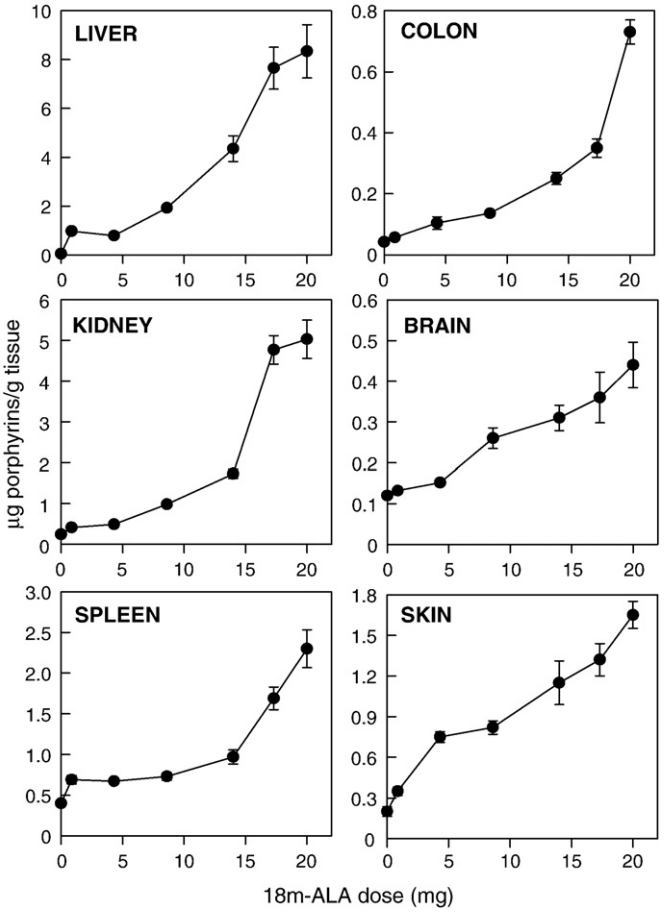

Fig. 4. Porphyrin accumulation in tissues after i.p. administration of increasing 18m-ALA doses. Different amounts of 18m-ALA were injected i.p. to mice. Twenty four hours later, tissues were excised and porphyrins extracted. Each data point represents the average of three determinations. Error bars show standard deviations.

the 18m-ALA dendrimer than from ALA itself at the same concentration. Porphobilinogen (PBG) accumulation was also two-fold higher for 18m-ALA. However, after 3 h incubation, not all the dendrimer had been hydrolysed to release ALA. Only 25% was released, giving rise to 25 pmol/10^5 cells of ALA, twice the amount accumulated from ALA itself, which correlates well with the higher porphyrin synthesis observed at this concentration using the dendrimer.

After 24 h incubation, the ALA level did not differ significantly from that measured at 3 h, whereas using 18m-ALA a 1.6-fold increase was found. PBG accumulation increased about 2-fold from ALA at 24 h and 8-fold from 18m-ALA compared to 3 h. The percentage of hydrolysed ALA from the dendrimer increased from 25% to 40% after 24 h exposure, thus releasing 64 pmol ALA/10^5 cells.

Following i.p. administration using a range of 18m-ALA doses, porphyrin production was measured 24 h later (Figs. 3 and 4). The maximum dose tolerated without symptoms of discomfort was 20 mg/kg. Porphyrins were synthesised in all the tissues analysed, including the tumour and the skin overlying the tumour, and the porphyrin levels increased with the administered dose. The highest levels were consistently found in the liver. The porphyrin content induced by the dendrimer was measured at 24 h since this time-point corresponded to maximal values measured in the pharmacokinetics studies as shown in Figs. 5 and 6, which also included comparisons with ALA.

ALA and 18m-ALA were then compared at equal drug equivalent doses, for porphyrin synthesis when administered i.p. to mice (Figs. 5 and 6). The kinetics of porphyrin generation induced by ALA clearly shows a sharp peak between 3 and 4 h in most tissues. In contrast, the 18m-ALA dendrimer induced sustained porphyrin production for over 24 h, and basal values were not reached until 48 h. In tumour and skin overlying the tumour, peak levels were found at 24 h (Fig. 5). Maximal porphyrin levels induced by the dendrimer were found in the liver with a peak at around 24 h, which was more pronounced than in other tissues where high levels were observed (spleen, kidney). In colon however, the 18m-ALA dendrimer was relatively inefficient compared to skin for porphyrin generation compared to ALA, with peak levels less than 10% of the porphyrin peak levels induced by ALA. Whereas in skin, peak levels induced by the dendrimer were only 50% lower than for ALA. A key observation is that the integrated porphyrin accumulation (ie areas under the porphyrin concentration traces) from 18m-ALA and an equal drug equivalent dose of ALA are comparable in Figs. 5 and 6, apart from the colon, which indicates that the majority of ALA residues are released from 18m-ALA and that total porphyrin production is comparable from both compounds.

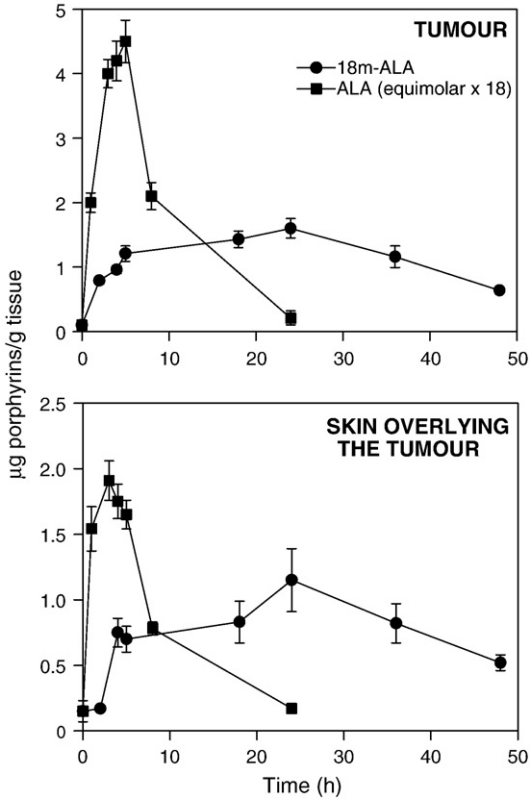

Fig. 5. Porphyrin synthesis from ALA or 18m-ALA in LM3 tumour and skin overlying the tumour as a function of time. Drug equivalent doses of ALA and 18m-ALA were injected separately i.p. to mice. At different times, tissues were excised and porphyrins extracted. Each data point represents the average of three determinations. Error bars show standard deviations.

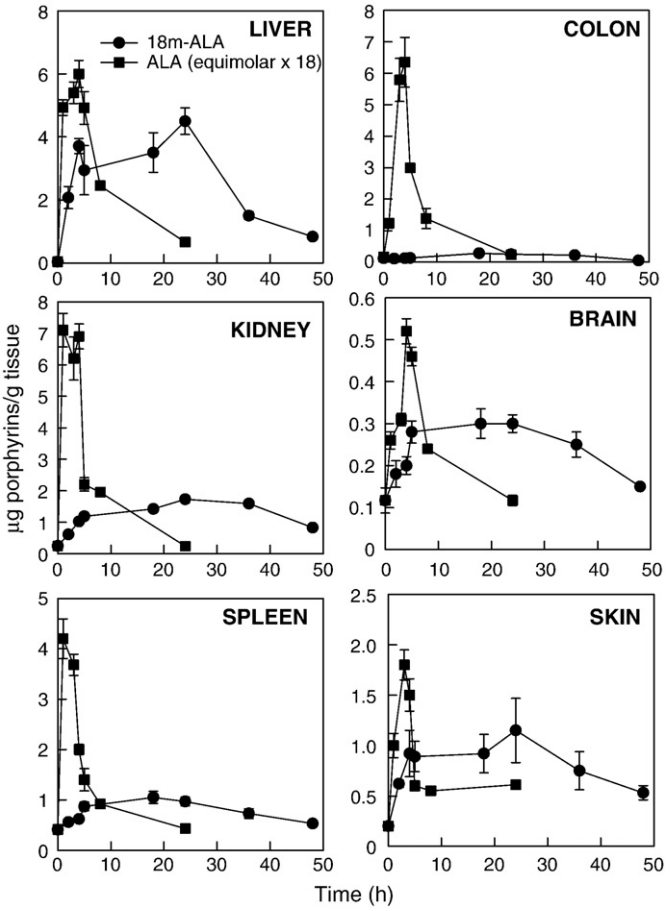

Fig. 6. Porphyrin synthesis from ALA or 18m-ALA in tissues as a function of time. Drug equivalent doses of ALA and 18m-ALA were injected separately i.p. to mice. At different times, tissues were excised and porphyrins extracted. Each data point represents the average of three determinations. Error bars show standard deviations.

4. Discussion

In this work the porphyrin synthesis induced by systemic administration of a dendrimer conjugate of 5-aminolaevulinic acid was examined in a subcutaneous murine tumour model and normal tissues. Unlike conventional photosensitising agents, the use of ALA-dendrimer conjugates for porphyrin generation and photodynamic therapy requires that the ALA residues are conjugated via cleavable linkages, in this case esters, so that the ALA can be liberated via enzymatic hydrolysis for subsequent conversion to porphyrin. The key advantages of using a dendritic delivery system are firstly the well-defined chemical structure of dendrimers, and secondly the chemical

functionality of the dendrimer end groups to which bioactive agents can be attached, thereby conferring a high 'payload' of the agent for each dendrimer moiety. The dendrimer investigated here (18m-ALA) incorporated eighteen aminolaevulinic acid residues attached via ester linkages to a multipodent aromatic core. We have previously examined this dendrimer in two cells lines [28], and showed that in PAM 212 murine keratinocytes and A431 human epidermoid carcinoma cells, the dendrimer was significantly more efficient compared to ALA for porphyrin synthesis. This correlated with higher cellular phototoxicity following light exposure, observed with the dendrimer. By using low temperature incubation and endocytosis inhibitors, we inferred that cellular uptake of the dendrimer (MWt of 3679) occurs through non-receptor mediated endocytic routes, whereas ALA is taken up via active transport [33]. The other significant finding was that following a short exposure of the cells to the compounds of 15–30 min duration, porphyrin generation was sustained over a much longer period, up to 48 h, compared to ALA which exhibited peak porphyrin production after 3–4 h, thereafter declining to basal levels. These results are consistent with gradual release of the ALA residue from the dendrimer following endocytic uptake. In the present study we have demonstrated that sustained porphyrin generation up to 48 h occurs *in vivo*. We have also obtained further corroboration for sustained porphyrin generation *in vitro* based on studies of the rate of intracellular release of ALA from the dendrimer (Table 1). The results obtained here using a murine mammary adenocarcinoma cell line, LM3, and an ALA-based assay instead of a porphyrin assay, confirm the slow intracellular release of the ALA from the dendrimer (Table 1). The rate of release of ALA residues (25% at 3 h and 40% hydrolysis at 24 h) shows that the esterases activities and/or the release of ALA from the endo/lysosomal compartment, can regulate the bioavailability of ALA. In our previous study [28] we proposed that steric hindrance as well as esterase availability limited the rate of ALA cleavage. We have previously demonstrated that at high concentrations ALA ester hydrolysis is regulated by esterases via end product regulation for esters [34] and small dendritic derivatives [27]. The *in vitro* data indicate that similarly, 18m-ALA hydrolysis is partly regulated by the rate of consumption of ALA to synthesise porphyrins. However as discussed later, the *in vivo* regulation could be completely different since 18m-ALA is administrated in a single dose and rapid uptake of the compound by tissues may result in different esterase/endosomal release regulation leading to a low and continuous ALA liberation.

The LM3 adenocarcinoma cell line was also used for the tumour model studies presented here. ALA is known from our previous studies to be efficiently metabolised to porphyrins in these cells, and a comparison was carried out versus the 18m-ALA dendrimer, which showed that the dendrimer was more efficient than ALA in terms of porphyrin synthesis both at short and long incubation times up to [0.1 mM] concentrations (Fig. 2). The enhancement in porphyrin production from 18m-ALA is due in part to the high ALA payload of the 18m-ALA dendrimer but also probably governed by the particular kinetics of uptake of the ALA dendrimer by these mammary adenocarcinoma cells. The decrease in porphyrin levels observed at higher dendrimer concentrations may be due to a combination of toxicity and decomposition in the medium, giving a polymeric degradation product [28]. The polymerisation was proposed to take place primarily via intermolecular condensation reactions between ALA residues, a process which readily occurs in neutral to basic ALA solutions.

Upon systemic i.p. administration, whereas the kinetics of porphyrins from ALA clearly show a sharp peak between 3 and 4 h in most tissues, 18m-ALA induced sustained porphyrin production for over 24 h, and basal values were not reached until 48 h. Comparing areas under the curves, ie the integrated porphyrin accumulation, we can conclude that the majority the ALA residues are released from 18m-ALA and that the total porphyrin production is the same from both compounds. The colon is the exception, with an area under the curve three times lower from 18m-ALA compared to ALA. Comparing the tumour versus the skin overlying the tumour levels there does not appear to be an enhancement of tumour selectivity over ALA. The main difference is that using ALA as discussed later, peak levels occur over a duration of 2–3 h, which in turn limits the optimum timing for PDT treatment. The porphyrin kinetics observed using ALA here at a dose of about 300 mg/kg agree with previous studies by Peng et al. [35] who used i.p. doses of 250 mg/kg in mice.

As expected, liver and spleen, which are organs of the reticuloendothelial system, together with kidney, exhibit the highest porphyrin accumulation from 18m-ALA, followed by tumour and skin. Likewise, they are also the major organs for porphyrin production from ALA itself. Brain porphyrin levels produced from 18m-ALA and ALA are low, which was expected due to the presence of the haematoencephalic barrier. A maximum dose of 20 mg 18m-ALA was tolerated after systemic injection to tumour bearing mice. Except for the colon, there was a correlation between the organs that synthesise more porphyrins from ALA and those that synthesise porphyrins from the dendrimer. This suggests that the accumulation of porphyrins depends on the efficiency of porphyrin conversion from ALA and that ALA or dendrimer uptake is not limiting, even in tumour tissue. The uptake mechanism of ALA and the dendrimer is markedly different; ALA is taken up by active transport [36], whereas we have previously found in PAM 212 cells that 18m-ALA is taken up predominantly by non-receptor mediated endocytosis [28]. However despite the different routes, the rate of cellular uptake of the dendrimers appears to be comparable to ALA [28]. In colon, ALA uptake might be particularly high probably due to the presence of basolateral peptide transporters, which recognise and transport ALA in intestinal cells [37], but low in its endocytic ability.

The *in vivo* data suggest that ALA is being gradually processed inside the cells to give rise to sustained porphyrin production which is consistent with the *in vitro* results reported here using an ALA-based assay and our previous study on porphyrin kinetics in other cell lines [28]. The data in Table 1 showing slow hydrolysis *in vitro* reinforce the hypothesis of slow release of ALA residues, regulated by either esterase activity or release from endo/lysosomal compartments. The ALA molecules available to synthesise porphyrins within the tissue could be either released from the dendrimer by the serum esterases or alternatively by intracellular esterases. In the first case, ALA uptake should govern porphyrin kinetics and thus all organs would present similar profiles, whilst in the second, endocytosis should be also involved. Based on the observation that low colonic porphyrins are formed from the dendrimer, we could hypothesise that ALA release by serum esterases is almost negligible and that ALA available to synthesis porphyrins come mainly from the intracellularly taken up 18m-ALA. This is probably due to relatively rapid cellular uptake of 18m-ALA leading to short half-life in circulation, as observed for other dendrimers of similar size (see below). It is relevant to note that the ALA half-life in circulation following iv administration is approximately 30 min [38]. The slight variations among the kinetics of the different tissues could thus be ascribed to their different endocytic abilities as well as processing the dendrimer to release ALA. It is also possible that ALA transporters [36] or receptors [39] might be retaining 18m-ALA attached to the cell surface thus modifying the uptake kinetics. In addition, although we cannot discard the contribution of spontaneous hydrolysis of 18m-ALA, and partial polymerisation due to inter/molecular reactions, and the different kinetic profiles of porphyrin accumulation in different tissues suggest again that factors unique for each tissue are crucial for the release of ALA.

The maximum dose of the dendrimer tolerated without symptoms of discomfort was 20 mg/kg. Toxicity of 18m-ALA may be due to the large number of exposed amino groups inducing non-specific binding, although we cannot exclude the contribution of toxic cleavage products affecting toxicity at high concentrations. The

cytotoxicity of cationic macromolecules, eg PAMAM dendrimers has been studied and reported extensively in the literature [21]. Their toxicity is proposed to result from the non-specific interaction between positively charged polymers and negatively charged cell membranes leading to a haemolytic effect [40,41]. The presence of primary amines exhibited a toxic effect on red blood cells, causing them to agglutinate [42]. This problem could in future work be avoided using by derivatising the amino groups although this would not be straightforward.

18m-ALA is a second-generation dendrimer with a molecular weight of 3679, which is too low to elicit the enhanced permeability and retention effect (EPR), which can improve tumour selectivity for larger dendrimers [43]. Several studies on cationic amino acid dendrimers of comparable molecular weight to 18m-ALA have been carried out [44–46], which demonstrated rapid clearance from the circulation following systemic administration. These studies would support our conclusion that the ALA dendrimer is cleared relatively rapidly from circulation and that the rate of cleavage of ALA and/or endosomal release within the cells is the rate-limiting step for porphyrin production. We have previously carried out *in vivo* studies of smaller dendritic ALA derivatives, ie G0 dendrons [27]. The kinetics of PpIX formation in tissues after i.p. administration of a dendron containing three ALA residues to tumour-bearing mice was comparable to that of ALA, suggesting that the liberation of ALA via deesterification did not significantly affect the rate of build up of tetrapyrroles from the dendron. However on an equimolar basis, the efficiency of porphyrin generation was lower compared to ALA itself. By the use of larger second-generation dendrimers, on the contrary, we appear to have achieved not only more complete ALA liberation *in vivo*, resulting in more efficient porphyrin generation, but also sustained porphyrin production over several hours. The differences in kinetics between the smaller dendritic derivatives and dendrimers will depend on many factors such as the mechanism of uptake, the time required for cleavage of eighteen as opposed to three ALA residues, and retention in cells. The uptake mechanism may be important since the larger dendrimers should be retained longer within the endo/lysosomal compartment, which will affect access to esterases, whereas the smaller dendrons appear to be taken up by a combination of active and passive diffusion [47].

In the comparison of different dendrimer-drug delivery systems, it is important to distinguish between drugs that are conjugated as opposed to merely being incorporated by encapsulation into dendrimer. The slow degradation of the complex can also give rise to prolonged release *in vivo*. There are a few studies of molecules attached to the dendrimer core by ester linkages which show gradual release of the drug in cell lines [48] and upon exposure to plasma [49], but to the best of our knowledge, there are no studies of biodistribution of the released drugs. A recent study using the encapsulation method is of relevance however to the present work, where Kojima et al. [50] investigated pegylated PAMAM dendrimers incorporating protoporphyrin IX in HeLa cells and showed that the complex could induce photocytotoxicity. PAMAM porphyrin dendrimers have also been used successfully for photochemical internalisation to enhance gene delivery [51]. Several other polymeric delivery systems for photosensitisers have been investigated where the sensitiser is covalently bound. Hamblin et al. [52] investigated peglylated polylysine polymer conjugates bearing multiple photosensitiser moieties, which were found to be an effective means of photosensitiser delivery to cells. Based on these studies, pegylation of the ALA-dendrimers is another area for investigation in future work.

In conclusion we have demonstrated a good correlation between *in vitro* and *in vivo* properties of an ALA-dendrimer conjugate which is able to induce sustained and efficient porphyrin production. Since ALA is metabolised within cells to a fluorescent product that can be directly detected, the study of ALA dendrimers may be a useful model for optimising the structure of other dendrimers conjugated with small chemotherapeutic agents. Although a sustained porphyrin level was obtained not only in tumour but also in the normal tissues analysed after 18m-ALA administration, this may present a therapeutic advantage in terms of dose fractionation and/or in combination with prolonged low level light delivery. The possibility of using dendrimers for sustained delivery of ALA should be investigated further in view of previous work using fractionated dosing of ALA where better tumour treatment was obtained [38,53]; moreover single bolus ALA dosing results in a greater immediate loss of ALA and its metabolite, porphobilinogen, into the urine [54]. Regarding prolonged irradiation regimes, the concept of 'metronomic PDT has been recently introduced to describe continuous low-dose administration of light and photosensitiser [55–57]. The low-level irradiation employed in metronomic PDT appears to favour apoptosis and thus relatively little inflammation, which is desirable in treating brain tumours. Several methods of light delivery have been proposed for irradiation over prolonged periods of several hours, but this in turn requires that the photosensitiser be administered either continuously or in fractionated doses. This approach should in principle be well suited to ALA-PDT using a dendrimer since only one dose of the dendrimer need be given in combination with a prolonged low intensity light regime tailored to the porphyrin kinetics. Bisland et al. [55] performed metronomic ALA-PDT in a rat model of glioma cells orthotopically implanted into normal brain after ALA administration via the drinking water for 5 days. The porphyrin values obtained after 5 days were 0.56 ± 0.12 μg porphyrins/g in normal brain and 2.16 ± 0.5 μg porphyrins/g in tumour. In the present work, we employed a model of subcutaneous implanted mammary tumour and the amount of porphyrins formed from 18m-ALA was around 1.6 μg porphyrins/g in tumour and around 0.3 μg porphyrins/g in brain, and those values were sustained from 2 h to 36 h after single bolus administration. It appears therefore that whereas comparable tumour porphyrin levels were observed in both studies, lower levels were observed in brain using the dendrimer. Further studies would be needed to assess the feasibility of the use of metronomic PDT with ALA dendrimers for the treatment of malignant brain tumours.

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