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NOTE ARTICLE

Doxorubicin conjugation and drug linker chemistry alter the intravenous and pulmonary pharmacokinetics of a PEGylated Generation 4 polylysine dendrimer in rats

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Abstract:

PEGylated polylysine dendrimers have demonstrated potential as inhalable drug delivery systems that can improve the treatment of lung cancers. Their treatment potential may be enhanced by developing constructs that display prolonged lung retention, together with good systemic absorption, the capacity to passively target lung tumours from the blood and highly selective, yet rapid liberation in the tumour microenvironment. This study sought to characterise how the nature of cathepsin B cleavable peptide linkers, used to conjugate doxorubicin to a PEGylated (PEG570) G4 polylysine dendrimer, affect drug liberation kinetics and intravenous and pulmonary pharmacokinetics in rats. The construct bearing a self-emolative diglycolic acid-V-Citrulline linker exhibited faster doxorubicin release kinetics compared to constructs bearing self emolative diglycolic acid-GLFG, or non-self emolative glutaric acid-GLFG linkers. The V-Citrulline construct exhibited slower plasma clearance, but faster absorption from the lungs than a GLFG construct, although mucociliary clearance and urinary elimination were unchanged. Doxorubicin-conjugation enhanced localisation in the bronchoalveolar lavage fluid compared to lung tissue, suggesting that projection of doxorubicin from the dendrimer surface reduced tissue uptake. These data show that the linker chemistry employed to conjugate drugs to PEGylated carriers can affect drug release profiles and systemic and lung disposition.

Keywords: pharmacokinetics, lung clearance, cathepsin B, pulmonary, doxorubicin, dendrimer.

Abbreviations: BALF, bronchoalveolar lavage fluid; DGA, diglycolic acid; VCit, Valine-Citrulline; Dox, doxorubicin; Glu-GLFG; glutaric acid-glycine-leucine-phenylalanine-glycine; GFLG, glycine-leucine-phenylalanine-glycine; DGA, diglycolic acid; PAB, para-amino benzoic acid; G, generation.

Introduction:

Previous work has shown that inhaled PEGylated polylysine dendrimers provide an excellent platform for the delivery of drugs to the lungs, and for subsequent systemic exposure[1, 2]. They exhibit tuneable pharmacokinetic properties that can be tailored to promote prolonged lung retention, systemic access or both[2]. For example, pulmonary administration of a 56 kDa PEGylated G5 dendrimer conjugated with the chemotherapeutic drug doxorubicin (Dox) via an acid labile hydrazone linker, dramatically improved the activity of doxorubicin against lung metastases in rats when compared to the intravenous administration of drug alone[1]. This construct was also well tolerated by rats after administration of up to 80 mg of dendrimer to the lungs. In contrast, the pulmonary administration of doxorubicin alone induced significant lung-related toxicity after a single dose. In spite of these encouraging results, some evidence of lung necrosis was apparent in ~50% of animals administered the Dox-dendrimer as a result of non-specific Dox liberation.

To enhance the therapeutic utility of this system, we sought here to explore the potential of conjugation chemistries to reduce the degree of non-tumour specific drug release, yet allow rapid drug liberation in tumours, and also to try to identify constructs that exhibit prolonged lung exposure, good systemic absorption and enhanced plasma exposure. Thus, we aimed to provide an inhalable delivery system with the potential to be retained in the lungs resulting in specific drug liberation on the 'air side' of lung tumours, together with absorption from the lungs and passive targeting from the 'blood side' of tumours via enhanced permeation and retention to enhance drug exposure to the whole tumour. In this way, pulmonary and systemic side effects are expected to be reduced (compared to drug alone) by minimising the exposure of non-cancerous tissues to free drug.

A series of G4 dendrimers conjugated with PEG570 and doxorubicin (20-30 kDa in size) were synthesised based on previous data showing that this scaffold construction offers both prolonged systemic exposure and good lung absorption[2, 3] (see figure in supporting information). Three peptide-based drug linkers that are specifically cleavable by cathepsin B were used to conjugate doxorubicin to this dendrimer scaffold: a pentapeptide (Glu-GLFG[4]) that results in the liberation of both peptide-modified and unmodified doxorubicin, and two self-emolative systems (diglycolic acid-GLFG-para-amino benzoic acid[4] and diglycolic acid-Valine-Citrulline (VCit)-para-amino benzoic acid[5]) that facilitate the liberation of unmodified doxorubicin. Cathepsin B-cleavable peptides were used since the extracellular and lysosomal expression of this enzyme is highly upregulated by cancer cells, particularly those at the invasive front of a tumour, but is constitutively expressed at low levels in normal tissue[6-8].

Methods:

Materials and dendrimers

Scintillation vials, Soluene tissue solubiliser and IRGASafe scintillant were from Perkin Elmer (Vic, Australia). Cell culture media and supplements were from Invitrogen (Vic Australia).

Dendrimers were synthesised and characterised as described previously[9], with modification (see supplementary information for synthetic details). ^{3}H-lysine was incorporated into the penultimate generation to enable pharmacokinetic characterisation of the dendrimer construct. The specific activity of the dendrimers was determined via scintillation counting[10].

Doxorubicin release kinetics and in vitro cytotoxicity

The kinetics of Dox release in the presence of cathepsin B was initially investigated by incubating 1 ug/ml Dox equivalents (approx. 5 ug/ml dendrimer) with 1 IU/ml cathepsin B (Sigma, MO, USA) in 0.1 M acetate buffer containing EDTA (0.01 M) and glutathione (0.05 M) at 37°C for 2 days. Dox release from $G4_{PEG570}$-DGA-VCit was also examined in the absence of cathepsin to confirm enzyme-specific liberation. Aliquots of the reaction mixture were collected at various time points and assayed via fluorescence HPLC for free Dox[11].

The in vitro growth inhibitory effects of the Dox dendrimers, as well as a solution formulation of Dox and a non-Dox conjugated control dendrimer were evaluated using an MTT assay in 96 well microplates against human mammary MCF7 cells and human lung A549 cells over 3 days and a concentration range of 0.1 nM to 500 uM as previously described[9]. Cells were grown in RPMI media supplemented with 10% FBS and 1% glutamax and maintained in a humidified environment at 37°C and 5% CO_2.

Intravenous and pulmonary pharmacokinetics in rats

Male Sprague Dawley rats (8 weeks) were obtained from the Monash Animal Platform (Monash University, Vic, Australia) and housed at ambient temperature on a 12 h light/dark cycle. Water and food were freely available with the exception of overnight after surgery and for up to 8h after dosing. All experimentation involving animals was approved by the institutional animal ethics committee.

The right carotid artery and jugular vein of rats was cannulated with 0.96 mm external / 0.58 mm internal diameter polyethylene catheters to facilitate IV dosing and serial blood sampling respectively under isoflurane anaesthesia[10]. After overnight recovery in individual metabolism

cages, rats were delivered a dendrimer dose of 5 mg/kg (in 1 ml saline) IV via the jugular vein cannula, or via intratracheal instillation into the lungs (in 150 ul saline) as previously described[2, 10]. Urine and feces were collected and assayed via scintillation counting during this time as previously described[10]. Serial blood samples (200 ul) were collected from the carotid artery cannula over 5 days, after which time rats were terminated and bronchoalveolar lavage fluid (BALF) and major organs collected for biodistribution analysis. A separate cohort of rats (n=3 per dendrimer) were dosed via the lungs with dendrimer and euthanised 1 or 9 days later and BALF and lung tissue collected to evaluate the time course of lung clearance.

The tritium content of plasma, urine, total feces, bronchoalveolar lavage fluid (BALF) and major organs (lungs, liver, kidneys, spleen, heart) was quantified via scintillation counting as previously described[10]. Non-compartmental pharmacokinetics were evaluated based on the specific radioactivity of dendrimers quantified in the plasma as previously described[12].

Size exclusion chromatography

Plasma, BALF and urine samples (100-200 ul; where sample radioactivity was sufficiently high) were injected onto a Superdex 200 column (GE Healthcare, Sweden) and eluted at a flow rate of 0.5 ml/min in a mobile phase of 50 mM PBS containing 0.3M NaCl (pH 3.5) to identify ^{3}H species in these samples. Fractions were collected at 1 min intervals, mixed with 2 ml IRGASafe and analysed on a scintillation counter.

Results and Discussion:

Drug release kinetics and in vitro cytotoxicity

Of the Dox-conjugated dendrimers examined, the drug was most efficiently released from the DGA-VCit linker, with nearly 50% Dox released over 2 days (Fig 1) compared to no quantifiable release in the absence of cathepsin (<5%, the limit of assay sensitivity; not shown). Approximately 33% of unmodified Dox was released from the DGA-GLFG linker over 2 days, whilst 26% of Dox was released from the non-self emolative Glu-GLFG counterpart, presumably reflecting the predominance of cleavage to form FG-modified drug.

The *in vitro* cytotoxicity of the constructs and doxorubicin alone was then evaluated against human lung (A549) and mammary (MCF) adenocarcinoma cells (Fig 1B & C respectively). Despite the differences in Dox release kinetics, there were no significant differences in the growth inhibitory effects of the three constructs against A549 or MCF7 cells. A non-Dox loaded control dendrimer exhibited no growth inhibitory effects over the concentration range examined, confirming that the cytotoxicity of the Dox constructs was related to drug liberation. It is possible however, that cathepsin release into the media was not high enough over the 3 day incubation period to exemplify differences in the sensitivity of the linkers to cleavage by this enzyme in a cell-based system.

Intravenous pharmacokinetics and biodistribution

Since the DGA-self emolative systems displayed the best in vitro drug liberation profiles, these constructs were then progressed into in vivo studies in rats and their intravenous and pulmonary pharmacokinetics evaluated. The dendrimer containing the DGA-VCit linker was cleared ~2 fold slower than $G4_{PEG570}$-DGA-GLFG after IV administration, although terminal half-lives (approximately 1.5 days) were similar, indicating reductions in volume of distribution of a similar magnitude (Fig 2, Table 1). In contrast, a corresponding fully PEGylated construct (ie non-drug conjugated, G4 100% PEG_{570} conjugated) reported previously, was cleared from plasma ~1.5 to 3 fold faster, although terminal half-life was similar (supporting information)[3]. These differences may reflect changes in the interaction of dendrimers with membranes and plasma proteins as a result of differences in the presentation or orientation of Dox which is extensively protein bound in plasma.

Despite the reduction in total clearance and volume of distribution of $G4_{PEG570}$-DGA-VCit, ~45% of both Dox-dendrimers were eliminated via the urine (Table 1)[12] and at the same rate (not shown). Although the proportion of urinary elimination was similar to the fully PEGylated dendrimer, ^{3}H-label from the Dox-dendrimers was eliminated as both intact dendrimer and low MW products of polylysine catabolism (supporting information), which was not observed previously for the fully

PEGylated construct. This suggests that the Dox-conjugated dendrimers exhibit faster rates of catabolism than the fully PEGylated construct, presumably as a result of the liberation of Dox over time and exposure of the polylysine scaffold to proteases[12]. Organ biodistribution profiles of the two dendrimers were also similar, although $G4_{PEG570}$-DGA-GLFG displayed moderately higher organ retention than $G4_{PEG570}$-DGA-VCit (Fig 3 and supporting information)[3]. Dox dendrimers also displayed more extensive kidney retention after IV administration than the fully PEGylated dendrimer, despite displaying similar urinary elimination (supporting information).

Pulmonary pharmacokinetics and biodistribution

After pulmonary administration of $G4_{PEG570}$-DGA-GLFG plasma concentrations plateaued over 1 to 5 days post dose at approx. 1000 ng/ml (Table 1). As a result, absolute bioavailability was not determined, although 12% of the dose was bioavailable up to 5 days (based on truncated AUC to 5 days). $G4_{PEG570}$-DGA-VCit was absorbed from the lungs with a Tmax of 30 h, and 9% of the dose was absorbed as intact dendrimer over 5 days. In this case, a terminal elimination half-life could be estimated (possibly due to more extensive urinary elimination after pulmonary administration) and therefore absolute bioavailability was calculated to be ~20%. In contrast, previous studies suggest that the fully PEGylated counterpart is absorbed faster than the Dox dendrimers examined here (Tmax 13 h; Cmax ~2600 ng/ml) and has higher pulmonary bioavailability (up to 30%)[2]. The presentation of Dox on the dendrimer surface therefore appears to slow and potentially limit pulmonary absorption.

Despite differences in the absorption profiles after pulmonary administration, lung clearance rates and extent of mucociliary elimination from the lungs (10-15%) were similar for both dendrimers (Table 1, Fig 3). The Dox dendrimers were also mainly localised in the BALF, whereas the previously reported fully PEGylated dendrimer was predominant localisation in lung tissue[3].

Conclusion

In summary, the results suggest that the presentation of Dox on the surface of a short PEG (570 Da)-conjugated G4 dendrimer prolongs retention in the lung mucus, and slows absorption from the lungs and plasma clearance compared to a fully PEGylated dendrimer. This is in contrast to larger (G5) dendrimers with higher PEG loading (PEG1100) which appear to better mask the drug in vivo[1]. The data further suggest that the dendrimer with the shortest $G4_{PEG570}$-DGA-VCit self emolative linker displays the most optimal balance between prolonged lung and plasma exposure, together with absorption from the lungs after pulmonary administration, whilst also providing maximal rates of drug release in the presence of cathepsin B.

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Figures:

Figure 1. Doxorubicin release kinetics and in vitro cytotoxicity. **Panel A:** Kinetics of Dox release from each dendrimer in the presence of cathepsin B. **Panel B:** Cytotoxicity of the three dendrimers against human lung carcinoma A549 cells, and **Panel C:** human mammary MCF7 cells. Symbols represent: (○) $G4_{PEG570}$-DGAVCit; (●) $G4_{PEG570}$-Glu-GLFG; (●) $G4_{PEG570}$-DGAGLFG; (□) Dox; (■) Blank dendrimer. Data represent mean ± s.d. (n=3).

Figure 2. Plasma concentration-time profiles for (A) $G4_{PEG570}$-DGA-GLFG, and (B) $G4_{PEG570}$-DGA-VCit after intravenous and pulmonary administration to rats at 5 mg/kg. Mean ± s.d. (n=3-4).

Figure 3. Biodistribution of dendrimers and time course for dendrimer clearance from the lungs after pulmonary administration. Organ biodistribution of (A) ^{3}H-$G4_{PEG570}$-DGA-GLFG, and (B) ^{3}H-$G4_{PEG570}$-DGA-VCit was determined 5 days after IV or pulmonary administration to rats. The time course for the lung clearance of (C) $G4_{PEG570}$-DGA-GLFG and (D) $G4_{PEG570}$-DGA-VCit was evaluated separately in BALF and lung tissue over 9 days after pulmonary administration. Mean ± s.d. (n=3-4).

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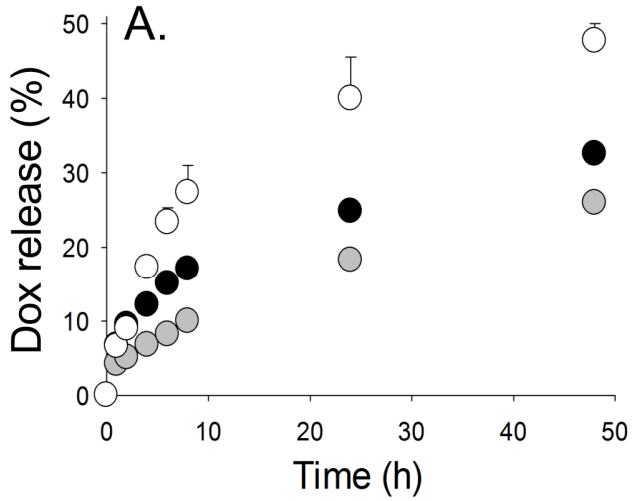
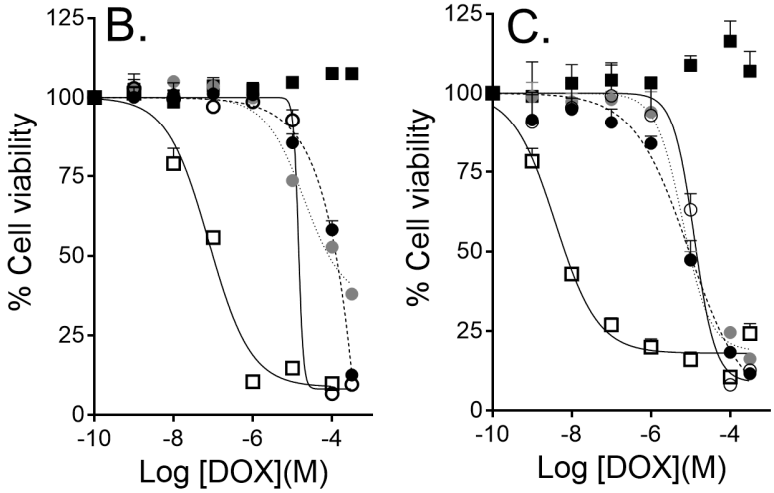
Table 1. Pharmacokinetic parameters of ^{3}H-dendrimers after intravenous and pulmonary administration to rats at 5 mg/kg. Mean ± s.d. (n=3-4). *p<0.05 cf. DGA-GLFG.

		$G4_{PEG570}$-DGAGLFG		**$G4_{PEG570}$-DGAVCit**	
Parameter	Units	**IV**	**Pulmonary**	**IV**	**Pulmonary**
$t_{½}$	h	33 ± 7	ND	36 ± 3	102 ± 60
$AUC_{0-\infty}$	µg/ml.h	686 ± 100	ND	1526 ± 119*	254 ± 95
AUC_{0-5d}	µg/ml.h	-	84 ± 21	-	131± 77
V_c	ml	14 ± 2	-	12 ± 1	-
$V_{D\beta}$	ml	87 ± 25	-	43 ± 6*	-
Cl	ml/h	1.8 ± 0.4	-	0.8 ± 0.1*	-
T_{max}	h	-	24 to 120	-	30 ± 0
C_{max}	ng/ml	-	999 ± 286	-	1639 ± 628
F (to 5d)	%	-	12 ± 3	-	9 ± 5
Urine	%	48 ± 5	8 ± 3	43 ± 10	16 ± 6
Feces	%	ND	15 ± 4	ND	10 ± 9

ND: not determined. BQ: below quantification.


A.
Dox release (%)
Time (h)
0
10
20
30
40
50


B.
C.
% Cell viability
Log [DOX](M)
125
100
75
50
25
0
-10
-8
-6
-4