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Doxorubicin-Conjugated PEGylated Dendrimers Show Similar Tumoricidal Activity but Lower Systemic Toxicity When Compared to PEGylated Liposome and Solution Formulations in Mouse and Rat Tumor Models

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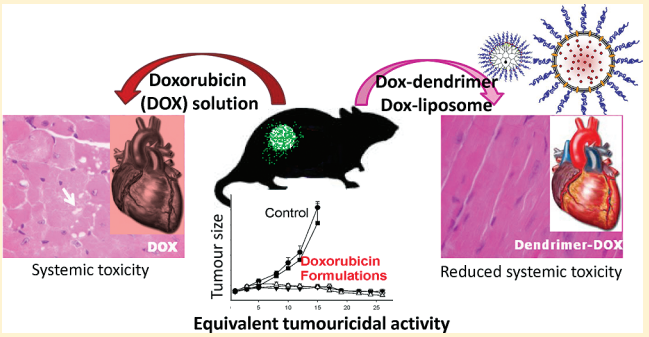
Supporting Information

ABSTRACT: PEGylated polylysine dendrimers show promise as novel drug delivery systems with the potential to direct site specific deposition patterns and to reduce toxicity at nontarget sites. Here the activity and toxicity profiles of a generation 5 polylysine dendrimer with 50% surface conjugation of PEG1100 and 50% surface conjugation of doxorubicin (via an acid labile 4-hydrazinosulfonyl benzoic acid linker) have been compared in a Walker 256 rat tumor model and a human MDA-MB231 xenograft in mice. A direct comparison was also made to a PEGylated liposomal formulation of doxorubicin and a doxorubicin solution. In both rat and mouse breast cancer models, the dendrimer formulation gave equivalent antitumor efficacy when compared to the liposomal or solution doxorubicin formulations and administration of all three doxorubicin formulations resulted in a significant reduction (>75%) in tumor growth in both models at doses ranging from 2 to 10 mg/kg doxorubicin equivalents. The dendrimer formulation, however, was better tolerated by both rats and mice, and approximately 2-fold higher doses were required to induce similar levels of toxicity (as assessed by organ weight, peripheral white cell counts, body weight and survival curves) when compared to administration of the doxorubicin solution or PEGylated liposomal doxorubicin. In rats the appearance of palmar plantar erythematosis (PPE), or hand foot syndrome, was also less evident after administration of dendrimer doxorubicin when compared to the liposome. Finally, even after administration to mice at 2-fold higher doses, dendrimer−doxorubicin resulted in a reduced incidence of cardiotoxicity when compared with a simple solution formulation of doxorubicin. The data suggest that dendrimer-based doxorubicin formulations may provide advantage over solution and liposomal formulations of doxorubicin via a reduction in systemic toxicity.



KEYWORDS: *doxorubicin, dendrimer, PEGylation, liposome, tumor*

BACKGROUND

The lack of specificity of most small molecule chemotherapeutics has led to growing interest in the use of site specific drug delivery systems to enhance drug concentrations at the target tissue and to simultaneously reduce nonspecific exposure to sites of potential toxicity. Selective tumor accumulation may be achieved by drug association with nanosized or colloidal drug carriers that are engineered to provide for extended systemic circulation times, usually via derivatization of the particle surface with hydrophilic polymers such as polyethylene glycol (PEG). These long circulating colloids accumulate in tumors due to the relatively high permeability of the vascular endothelium in rapidly growing tumors (which allows improved access of nanoparticulate materials when compared to other extravascular locations) and the reduced efficiency of lymphatic drainage from many tumors

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(which delays clearance of nanoparticles from the tumor tissue). This is the enhanced permeation and retention (EPR) effect and has been described for nanoparticulate systems including liposomes, dendrimers, micelles and polymers.[1−8] Liposomal and protein aggregate-based delivery systems that employ EPR have been successfully introduced into the clinic as tumor targeted drug delivery systems and include liposomal formulations of doxorubicin (Doxil/Caelyx and Myocet) and a nanoparticulate albumin-conjugated paclitaxel (Abraxane). In addition to potential improvements in tumor biodistribution, EPR also reduces the concentration of free drug (i.e. non-nanoparticle associated) in the blood, thereby limiting systemic toxicity and side effects, and in practice much of the advantage of cytotoxic nanomedicines comes from their improved safety profile.[9,10] Presentation of drug to cancer cells as a nanoparticulate complex has also been suggested to minimize resistance associated with cellular efflux by enabling cellular internalization via alternate mechanisms.[11−15]

Despite the potential benefits of the currently marketed liposomal doxorubicin delivery systems, problems remain. For example, cardiotoxicity is still evident, although at a reduced frequency,[16,17] and Doxil/Caelyx use is commonly associated with the development of palmar plantar erythematosis (PPE). PPE is believed to reflect the gradual accumulation and nonspecific release of doxorubicin in the extremities, and manifests as a painful swelling of the hands and feet that ultimately requires a reduction in the administered dose and therefore less effective treatment.[18] The limitations of the current treatments have stimulated interest in alternate approaches to doxorubicin delivery via EPR. For example, a micellar formulation of doxorubicin (NK911) has been evaluated and shown to reduce the in vivo incidence of toxicity, and to provide improved chemotherapeutic activity when compared to a simple solution formulation of doxorubicin.[19,20] The micellar system also demonstrated improved tumor penetration in an in vitro tumor spheroid model when compared to Doxil,[4] presumably due to the small particle size of the micellar formulation (∼40 nm).

Dendritic polymers or dendrimers provide an alternate template for the construction of nanoparticulate drug delivery systems. Dendrimers have the advantage of small particle size (∼10−20 nm) and therefore potentially improved tumor penetration and also the inclusion of tumor specific drug release mechanisms. The latter usually comprise a linker inserted into the dendrimer structure between the dendrimer core and the drug that is cleaved under tumor specific conditions. The release trigger is commonly the presence of an enzyme that is overexpressed in the tumor microenvironment (e.g. MMPs[3,21,22] or cathepsin[23,24]) or changes to pH (since most tumors become hypoxic and acidic over time). The use of tumor specific drug release mechanisms has the advantage of further reducing the likelihood of nonspecific release at nontumor sites and therefore the potential for lower systemic toxicity.

Previously, we have described the pharmacokinetics, biodistribution and antitumor efficacy of a PEGylated polylysine dendrimer-based delivery system for doxorubicin (∼12 nm) and have compared this to the in vivo behavior of doxorubicin and doxorubicin encapsulated within a PEGylated liposome (L-DOX).[25,26] In the dendrimer-based system, doxorubicin was conjugated to the dendrimer surface via an acid labile hydrazone linker to enable drug liberation in the acidic microenvironment of solid tumors (Figure 1). In these

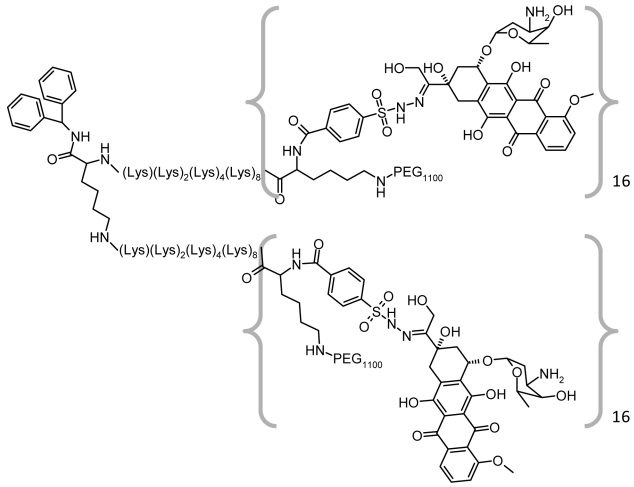

Figure 1. The generation 5 PEGylated polylysine dendrimer (D-DOX) contained PEG1100 conjugated to ε-amino groups on the terminal lysine layer and 4-hydrazinosulfonyl benzoic acid (HSBA)-doxorubicin conjugated to α-amino groups. Doxorubicin conjugated in this way has been shown previously to be liberated preferentially in acidic environments.[26] The structure shown represents the synthetic target where the efficiency of surface conjugation of PEG and doxorubicin is 100%. In practice the efficiency of conjugation was less than 100% and therefore the target structure does not provide the exact structure of the final constructs. The exact conjugation efficiencies are given in Methods and in the Supporting Information.

previous studies the dendrimer showed comparable tumor biodistribution and reduced uptake into the liver and spleen when compared to the liposomal formulation. Preliminary activity studies were subsequently conducted in a rat Walker 256 carcinoma model. At relatively low doxorubicin equivalent doses (2 mg/kg doxorubicin equivalents) no significant differences in tumor regression were apparent between the dendrimer, liposome and doxorubicin formulation, although all led to significant differences in tumor size relative to the untreated control. Preliminary evidence of reduced toxicity of the dendrimer formulation, however, was apparent, providing justification for a more detailed evaluation of the in vivo efficacy and toxicity of the doxorubicin constructs. Here, we report a more comprehensive evaluation of the antitumor efficacy and systemic toxicity of the dendrimer-based delivery system compared to doxorubicin encapsulated within a PEGylated liposome (L-DOX) and a simple solution formulation. More specifically, we (i) compare activity and toxicity profiles in two different tumor models, a mouse adenocarcinoma (MDA-MB231) and rat carcinoma (Walker 256) model; (ii) determine differences in the maximum tolerated dose (MTD) in the MDA-MB231 mouse model; (iii) evaluate the dose dependency of activity and toxicity in the rat model; (iv) extend the period of treatment for up to 4 weeks in rats and mice; (v) evaluate the potential for rebound growth for a period of 1.5 weeks (mouse) and 2 weeks (rat) after cessation of dosing and (vi) in the rat model track the appearance of PPE following administration of all delivery systems in addition to body weight, organ weights and white blood cell counts. In all cases, significant reductions in tumor growth were evident for all treatment groups, however the toxicity profile (as evidenced by changes to body weight, WBC counts, and appearance of PPE) was lower after administration of the dendrimer-based system.

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METHODS

Reagents. Doxorubicin HCl, Medium 199, Hanks balanced salt solution (HBSS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), fetal bovine serum (FBS) and RPMI were from Sigma (NSW, Australia). Glutamax, horse serum, penicillin/streptomycin, Matrigel, HBSS and trypsin-EDTA were from Invitrogen (VIC, Australia). Sterile saline was from Baxter Healthcare (NSW, Australia).

Doxorubicin Formulations. The doxorubicin HCl solution formulation was prepared as described previously[26] to provide a stock solution of 2 mg/mL doxorubicin HCl. Dosing solutions were diluted with 50 mM phosphate buffered saline (PBS, pH 7.4) as appropriate to provide a final volume of 500 μL and a dose of 2−8 mg/kg (rats) and a final volume of 100 μL/10 g body weight and dose of 3−5 mg/kg (mice). The doxorubicin solution formulation is referred to throughout the manuscript as DOX. Commercially available liposomal doxorubicin (Caelyx, Schering-Plough, NSW, Australia) consisted of doxorubicin incorporated into PEGylated phosphatidylcholine liposomes. This was supplied as a 2 mg/mL dispersion of liposomal doxorubicin HCl. The liposomal formulation was freshly aliquotted into sterile PBS on the day of dosing to provide a dose of 2−8 mg/kg in rats and 5−7 mg/kg in mice. The liposomal doxorubicin formulation is referred to here as L-DOX

Preparation of Dendrimer−Doxorubicin. The doxorubicin conjugated polylysine dendrimer (fifth generation) was synthesized as previously described.[26] The batch of material used to support the mouse studies (batch 1) was identical to that reported previously where 18 out of 32 available amine sites were PEGylated and 15 out of 32 amine sites contained hydrazone-doxorubicin, in turn giving a doxorubicin loading of 20% w/w. Rat studies were conducted with a second batch of material (batch 2) where longer reaction times between the dendrimer and the PEG_{1100} NHS ester resulted in improved surface coverage of PEG (see Supporting Information) (30 out of 32 available amine sites were PEGylated). The second batch had comparable (15 out of 32) numbers of amine sites populated with hydrazone-doxorubicin, but due to the increased molecular size of the construct (as a result of increased PEGylation) doxorubicin loading was reduced to 15% w/w (see Supporting Information for details of dendrimer characterization). PEG1100 was attached to surface ε-amino groups, and doxorubicin was linked through an acid labile 4-hydrazinosulphonyl benzoic acid (HSBA) linker through surface α-amino groups (SPL-8181).[26] The molecular weight of the second batch of dendrimer was 56.4 kDa and the diameter was 10.8 nm (polydispersity index was 0.16) as determined by photon correlation spectroscopy (Malvern Instruments, Worestershire, U.K.). A comparison of the physicochemical and pharmacokinetic properties of the two batches of dendrimer is given in the Supporting Information. Consistent with the previously reported relationship between molecular weight and clearance for PEGylated polylysine dendrimers[27] the clearance of the slightly larger and more highly PEGylated batch 2 construct was lower and the half-life slightly longer than those of batch 1. The doxorubicin conjugated dendrimer was lyophilized and stored at −20 °C. Prior to use, the dendrimer was resolubilized to 2 mg/mL doxorubicin equivalents in sterile PBS and then diluted with PBS to generate doses of 2−8 mg/kg in 500 μL (rats) and 8−12 mg/kg in 100 μL/10 g body weight (mice). The doxorubicin conjugated dendrimer is referred to throughout as D-DOX.

Animals. Athymic nude rats (male, 4−5 weeks) and athymic Balb/c mice (female, 7−13 weeks) were obtained from the Animal Resources Centre (WA, Australia). For the second mouse study, mice were obtained from the University of Adelaide Laboratory Animal Services (SA, Australia). Animals were housed on a 12 h light/dark cycle and were provided food and water at all times. All experiments involving rats were approved by the Victorian College of Pharmacy Animal Ethics Committee (Monash University, VIC, Australia), and all experiments involving mice were approved by the institutional Animal Ethics Committee at the Peter MacCallum Cancer Centre.

Cell Culture and Induction of Tumors. Walker 256 carcinoma cells were obtained from ECACC (Salisbury, U.K.). MDA-MB231 cells were obtained from ATCC (Manassas, VA, USA). Both cell lines tested negative for *Mycoplasma* contamination. Walker 256 cells were maintained in M199 media supplemented with 5% horse serum, 2 mM glutamine (as Glutamax) and 1% penicillin−streptomycin. MDA-MB231 cells were maintained in RPMI supplemented with 10% FBS, 2 mM glutamine (as Glutamax) and 1% penicillin−streptomycin. Both cell lines were kept at 37 °C in a humidified incubator at 5% CO_2. Cells were passaged by trypsin−EDTA digestion twice per week.

Walker 256 tumors in rats were induced by subcutaneous injection of $4-6 \times 10^6$ cells (in a volume of 200 μL of HBSS) into the right flank as previously described.[25] MDA-MB231 tumors in mice were induced by subcutaneous injection of 3×10^6 cells in 1:1 PBS:Matrigel into the right flank in a final volume of 100 μL. Animals were monitored daily, and tumor volume and body weight was measured every 1−3 days with calipers. In all cases, tumors were allowed to grow to approximately 100 mm^3 before initiating treatment.

Dosing Protocol in Rats. Treatments comprised 5 rats in each group, except for the group administered the highest dose of doxorubicin (8 mg/kg), in which only 2 rats were dosed per group. Only 2 rats were treated per group at this dose since this dose was lethal within 2 weeks. Rats were dosed with 2, 4, or 8 mg/kg doxorubicin equivalents of D-DOX, L-DOX or DOX twice per week for up to 4 weeks via a lateral tail vein in a final volume of 500 μL. Control animals received injections of 500 μL of sterile PBS or an equivalent dose of "empty" dendrimer that was conjugated only with the PEG and the HSBA linker. After the 4 week dosing period, surviving rats were allowed to recover and tumor growth was monitored continuously until animals were euthanized according to institutional termination protocols. Animals were humanely killed once an ethical end point was reached according to Australian Animal Ethics regulations. Specifically, this was the time at which any of the following conditions were met: tumor volume reached 10% of body weight, body weight decreased by 15% over a period of several days or more, animals reached grade 4 in the 4 point skin toxicity scoring system reported in the Supporting Information (indicative of palmer plantar erythematosis, PPE) or when consumption of food and water became difficult due to an observed impairment in motor coordination in some rats receiving high doses of doxorubicin. Euthanasia was via intravenous injection of 1 mL of Lethabarb under isoflurane anesthesia.

Determination of Maximal Tolerated Dose (MTD) of Doxorubicin Formulations in Mice. Thirty-nine mice were

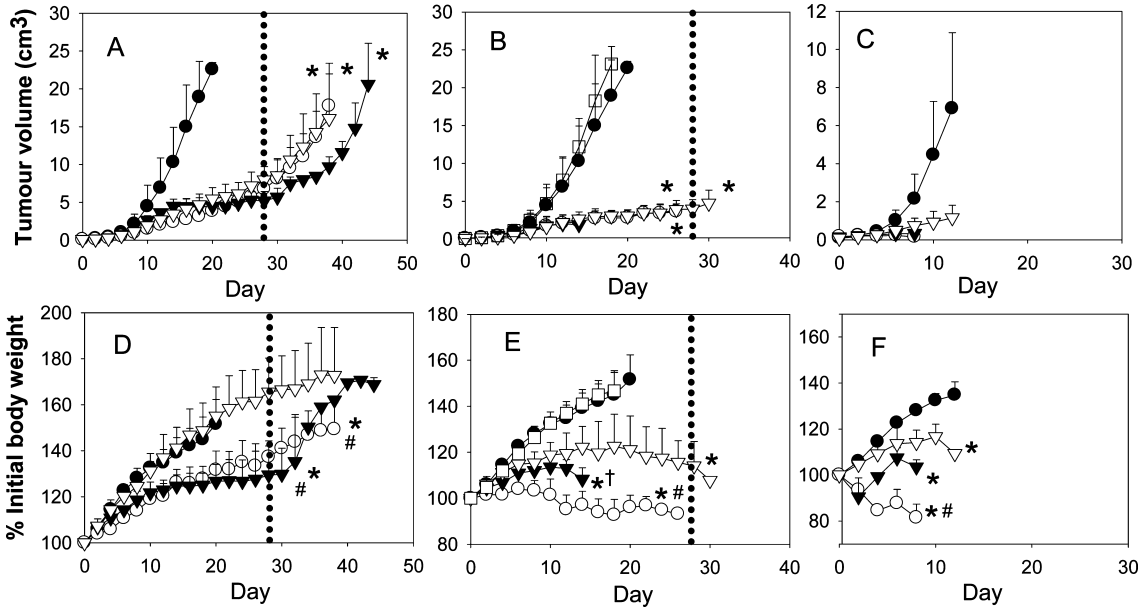

Figure 2. Measured growth curves of rat Walker tumors (upper panels) and body weight changes that exclude tumor mass (lower panels) at 2 mg/kg (A, D), 4 mg/kg (B, E) and 8 mg/kg (C, F) doxorubicin equivalents of PBS control (●), DOX (○), L-DOX (▼), D-DOX (▽) and control dendrimer (□). Data represent mean ± SD (n = 5 rats at the start of dosing). Symbols denote statistical differences in curve trend where * indicates significant difference compared to PBS, # indicates significant difference compared to D-DOX, † indicates significant difference compared to DOX. Dotted line indicates the day the final dose was given to the rats. At >32 days, only n = 2 rats in the L-DOX group remained as 3 rats required euthanasia due to the development of severe PPE.

separated into 13 groups with 3 mice in each group. Mice in each group were intravenously administered (via a lateral tail vein) one of the following formulations twice per week for 2 weeks, and surviving mice were then allowed to recover for up to 19 days: (i) PBS control, (ii) doxorubicin free dendrimer control (53 mg/kg), (iii) DOX (3 mg/kg), (iv) DOX (4 mg/kg), (v) DOX (4.5 mg/kg), (vi) DOX (5 mg/kg), (vii) L-DOX (5 mg/kg), (viii) L-DOX (5.5 mg/kg), (ix) L-DOX (6 mg/kg), (x) L-DOX (7 mg/kg), (xi) D-DOX (8 mg/kg doxorubicin equivalents), (xii) D-DOX (10 mg/kg doxorubicin equivalents), (xiii) D-DOX (12 mg/kg doxorubicin equivalents). Body weight was measured every day during the observation period. The maximal tolerated dose (MTD) for each doxorubicin formulation (DOX, 4.5 mg/kg; L-DOX, 5 mg/kg; D-DOX, 10 mg/kg doxorubicin equivalents) was determined as the dose at which mice lost no more than 10% body weight over any 5 day period during treatment and where body weight returned to baseline, predose levels during the recovery period.

Dosing Protocol in Mice. A preliminary study was initially conducted in mice to examine the antitumor efficacy of each doxorubicin formulation at the MTD and the resulting effects on organ weight. The treatment groups consisted of 4−5 mice which received twice weekly dosing of PBS, nondrug conjugated dendrimer control (43 mg/kg dendrimer) or DOX, L-DOX and D-DOX at the MTD for 3 weeks and mice were allowed to recover for up to 8 days after the last dose (see Supporting Information). Animals were humanely killed once an ethical endpoint was reached (as determined above for rats) or on day 26 and major organs collected and weighed. For the second study, treatment groups consisted of 4−7 mice in each group. Mice in each treatment group were administered PBS or DOX (4.5 mg/kg), L-DOX (5 mg/kg doxorubicin equivalents) and D-DOX (10 mg/kg doxorubicin equivalents) on days 2, 5, 9, 12, 22 and 26 after mean tumor volume reached 75 mm^3. Mice were allowed to recover for 10 days after dosing on day 12 and tumor regrowth monitored before administering further doses on days 22 and 26. Animals were humanely killed once an ethical end point was reached (as determined above for rats) or on day 29. Heart tissue was collected and stored in 10% formalin prior to histological analysis for toxicity by Gribbles Pathology Services.

Analytical Markers of Systemic Toxicity. Markers of systemic doxorubicin toxicity included reduced body and spleen weight, anemia, myelosuppression, palmar plantar erythematosis (skin toxicity, see Supporting Information for skin toxicity scoring system), impaired motor coordination and cardiotoxicity. Blood was collected via a lateral tail vein in Isoflurane anesthetized rats on days 1 (prior to the first dose), and on days 14, 28, and 42 or immediately prior to euthanasia. The white cell count in whole heparinized rat blood was determined by Gribbles Pathology. After euthanasia, liver, spleen, heart, lungs, skin and tumor were collected and weighed. Blood was collected from mice via ocular bleeds on days 1, 8, 15, 24, and 29 after tumors reached 75 mm^3 and analyzed for white blood cell counts by Gribbles Pathology Services. Hematocrit was also measured on terminal bleeds.

Statistics. Statistical analyses were performed using Graphpad Prism V4 (La Jolla, CA, USA). Within each dosing group (2, 4, or 8 mg/kg doses) survival curves for rats administered each of the doxorubicin formulations or PBS were compared using the Kaplan−Meier method. P values were extrapolated using the Log-rank (Mantel−Cox) test. Tumor growth curves and changes in body weight over time were examined separately for each dose (2, 4, or 8 mg/kg) for both rats and mice administered the doxorubicin formulations or controls were compared for statistical differences using a two way ANOVA with repeated measures to examine the effect of treatment group on tumor growth over time. Bonferroni post hoc tests were performed to identify least significant differences

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across dosing groups at each time point. Terminal spleen weight in rats and mice and white cell counts in rats were compared via one way ANOVA followed by a Tukey's post hoc test for least significant differences. White cell counts in mice were compared for statistical differences using a two way ANOVA and with a Bonferroni post hoc test to identify least significant differences across dose groups at time points. The incidence of cardiotoxicity in mice administered PBS or either of the 3 doxorubicin formulations was compared via logistic regression analysis (pairwise) with a Bonferroni adjustment. Significance was determined at a level of $p < 0.05$.

RESULTS

Comparative Activity versus Rat Walker 256 Tumors at 2 mg/kg, 4 mg/kg and 8 mg/kg Doxorubicin Equivalents. At a dose of 2 mg/kg all doxorubicin preparations (DOX, L-DOX and D-DOX) led to significant suppression of tumor growth when compared to administration of PBS (Figure 2A). The degree of growth suppression was approximately 70% at 14 days consistent with previous studies in the same tumor model.[25] The similarity in tumor growth suppression obtained with D-DOX in the current and previous studies[25] suggests that the relatively moderate differences in pharmacokinetic profiles of the different batches of material used to support the two studies (see Supporting Information) had little impact on pharmacodynamics for these constructs. The degree of growth suppression could not be quantified beyond 20 days since all control animals were euthanized at this point due to excessive tumor growth (tumor mass >10% body weight). From 14 to 28 days tumor growth was inhibited in all doxorubicin treated groups, but growth resumed on cessation of treatment at 28 days. A slight delay in the onset of tumor regrowth was evident in the L-DOX group (albeit for only $n = 2$ animals at time periods beyond 30 days, since others in the group were euthanized due to the onset of toxicity). This is consistent with previous studies where tumor retention of L-DOX was higher than that of D-DOX after repeated administration of 2 mg/kg doxorubicin equivalents.[25]

Although DOX, L-DOX and D-DOX were similarly able to suppress the growth of Walker 256 tumors, significant differences in systemic toxicity were evident on administration of the three different preparations at the low dose. Thus, L-DOX and DOX resulted in significantly slowed rat growth rates over a 10−30 day post dose period. In contrast, no significant differences were evident in the rate of growth in either control animals or animals administered D-DOX (Figure 2D). Furthermore, in all animals administered L-DOX, PPE (identified initially by the development of dry, flaky red skin on the ventral aspect of the body) was evident with varying levels of severity (see Supporting Information for skin toxicity scoring system) at termination (Figure 3), but was sufficiently significant (grade 4 skin toxicity, see Supporting Information) to require euthanasia of 3/5 animals by day 32. Importantly, PPE was not evident in any other dosing groups at the 2 mg/kg dose. All other toxicity markers were similar across all treatment groups (Table 1, Figure 4D). White blood cell (WBC) counts increased very rapidly in control animals, presumably as a function of tumor growth, and were approximately 5-fold higher at the point of euthanasia than at day 1 (Figure 4D). In all other groups, the increase in WBC counts was delayed due to the myelosuppressive actions of doxorubicin, but at 28 days was not significantly different from that at 14 days in the PBS group. Similarly, spleen weights were reduced by approximately

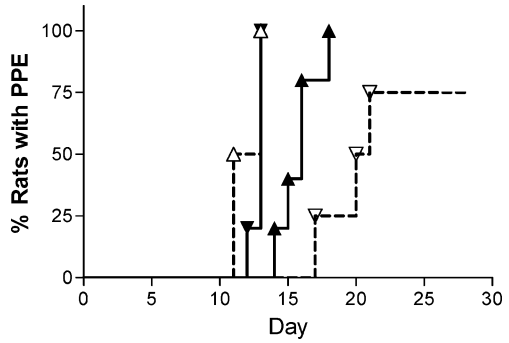

Figure 3. Onset of PPE in rats given 2 mg/kg L-DOX (▲), 4 mg/kg D-DOX (▽), 4 mg/kg L-DOX (▼), 8 mg/kg D-DOX (△). PPE was not evident in any of the other dosing groups. The number of animals in each group was 5 (except the 8 mg/kg D-DOX group where $n = 2$), such that 100% response reflects response in all 5 rats.

50% in all doxorubicin dosed animals when compared to control animals, however no differences between doxorubicin dosed groups were apparent (Table 1). Heart weights in the DOX group were also significantly heavier than in the other treatment groups. No significant differences in the weights of the other major organs (liver, kidney, lung) were evident in any of the groups (Table 1). Survival curves are depicted in Figure 4A and represent the combined effects of animal deaths due to ineffective treatment (where animals were euthanized due to tumor size exceeding 10% body weight), or in some cases significant drug-related toxicity. In the PBS (control) group all animals were euthanized by day 22 due to increases in tumor mass to ≥10% of body weight. In L-DOX treated animals, 3/5 animals were euthanized by day 32, due to complications associated with PPE. Although the final two animals also showed signs of PPE, this was not sufficient to warrant euthanasia. The additional 2 animals were subsequently euthanized at day 44 and day 46 due to tumor regrowth beyond 10% body mass after treatment was stopped. In the D-DOX group, all animals were euthanized between day 30 and day 46 due to rebound tumor growth or ulceration after treatment cessation.

As the dose was increased to 4 mg/kg the degree of tumor growth suppression was enhanced, and at 14 days approximately 80% suppression was evident compared to control animals, although again no differences were seen across the doxorubicin dosed groups (Figure 2B). A control dendrimer formulation was also administered at the same dendrimer dose (i.e. a mass of doxorubicin-free dendrimer equivalent to that administered in the 4 mg/kg doxorubicin equivalent D-DOX group). Administration of the non-drug conjugated dendrimer showed no significant differences in either activity or toxicity when compared to PBS, providing evidence of the lack of acute toxicity or activity of the dendrimer scaffold. Although tumor suppression was greater in animals administered 4 mg/kg doxorubicin equivalents, this was also accompanied by significant increases in systemic toxicity. Indeed tumor regrowth beyond 28 days could not be monitored as essentially all animals were euthanized due to toxicity end points prior to 30 days. Nonetheless, some differences in toxicity and toxicity patterns were evident across the doxorubicin groups. Thus, animals administered L-DOX very rapidly developed PPE and this necessitated euthanasia within 14−16 days (Figure 3). Animals administered DOX also showed significant evidence of generalized toxicity including emaciation and muscle wasting,

Table 1. Terminal Organ Masses (g) in Walker 256 Tumor Bearing Rats Administered Twice Weekly Doses of Doxorubicin at 2, 4, or 8 mg/kg/dose for up to 4 Weeks[a]

		2 mg/kg dose			4 mg/kg dose			8 mg/kg dose		
	control	DOX	L-DOX	D-DOX	DOX	L-DOX	D-DOX	DOX	L-DOX	D-DOX
liver	11.68 ± 0.67	11.36 ± 1.60	9.64 ± 2.35	10.84 ± 1.20	4.93 ± 1.27*	6.52 ± 1.19*	6.91 ± 1.31*	4.51/4.18*	4.99/4.19*	5.68/5.38*
kidney	1.93 ± 0.07	2.03 ± 0.11	1.87 ± 0.28	2.04 ± 0.12	1.27 ± 0.08*#	1.42 ± 0.08*	1.47 ± 0.17*	0.86/0.88*	0.94/0.71*	1.26/1.04*
heart	1.08 ± 0.12	1.20 ± 0.12#†	0.88 ± 0.12	0.93 ± 0.12	0.71 ± 0.14*	0.75 ± 0.13*	0.72 ± 0.08*	0.40/0.42*	0.55/0.38*	0.58/0.64*
lung	2.10 ± 0.27	2.13 ± 0.54	2.18 ± 0.38	2.25 ± 0.50	1.06 ± 0.36*#	1.43 ± 0.46	1.77 ± 0.42	0.92/1.15*	1.26/0.89*	0.73/0.95*
spleen	1.32 ± 0.33	0.71 ± 0.08*	0.48 ± 0.22*	0.66 ± 0.15*	0.11 ± 0.03*#	0.17 ± 0.03*#	0.26 ± 0.05*	0.06/0.05*#	0.09/0.07*	0.11/0.1*

[a]Rats were euthanized at specified end points according to institutional animal ethics protocols. Data represent mean ± SD (n = 5 rats) except for the 8 mg/kg dose which indicates the individual organ weights for each rat. * indicates significant difference compared to PBS control rats. # indicates significant difference compared to D-DOX. † indicates significant difference compared to L-DOX.

dry skin, narrowing of eye slits, labored breathing, lethargy, pale appearance, local tissue necrosis at tail vein injection sites and gastrointestinal disturbances (diarrhea). Rats administered D-DOX, however, displayed more prolonged survival times than rats administered PBS, DOX or L-DOX and were eventually euthanized around day 30 due to a combination of the loss of 15% body weight (Figures 2E and 4B) or the loss of motor coordination that began to affect eating, drinking and walking. This loss of motor coordination has been previously observed for high doses of doxorubicin.[28−31] Organ weights were also suppressed in all treatment groups (Table 1) although spleen weights were higher at the point of euthanasia in the D-DOX group when compared with L-DOX or DOX. Similarly, although WBC counts were suppressed in all treatment groups, they were higher at sacrifice in the D-DOX administered animals when compared with DOX or L-DOX. Preliminary histological investigation of cardiac tissue in rats administered the higher doses of DOX revealed a lack of consistent cardiotoxicity. Cardiotoxicity was therefore not investigated further in rats. Due to an expectation of significant drug related toxicity at 8 mg/kg only 2 animals per group were administered drug at this dose level. As expected, rapid suppression of tumor growth was apparent (Figure 2C), but toxicity was highly significant (Figures 2F and 4F) and all animals were euthanized by day 16 due to the onset of doxorubicin toxicity (Figure 4C).

Comparative Activity versus Human MDA-MB231 Tumors in Mouse Xenograft Model after Administration at MTD. The rat data in the current study extends previous studies using the Walker 256 model and confirms similar in vivo tumoricidal data for either dendrimer-based (D-DOX) or liposome-based (L-DOX) nanoparticulate delivery systems, but suggests potential toxicity advantages for the dendrimer-based delivery systems. As a continuation of these findings, the tumoricidal activity of the different delivery systems was subsequently evaluated in a human breast cancer cell line (MDA-MB231) and in a mouse xenograft rather than a rat model. These studies were conducted in three separate parts. First, the maximum tolerated dose for D-DOX was evaluated and compared with that of L-DOX and DOX (see Supporting Information). Second, a preliminary in vivo tumor activity study was conducted over 3 weeks which also allowed evaluation of simple toxicity parameters including organ weights across the different groups at sacrifice. Finally, a more comprehensive study was undertaken to evaluate the activity of D-DOX, L-DOX and DOX after dosing at the MTD in the MDA-MB231 tumor model and to evaluate toxicity and in particular the potential for cardiotoxicity, bearing in mind the significant clinical cardiotoxicity that is associated with doxorubicin administration.

To determine the MTD, animal weights were monitored daily after twice weekly iv dosing of each of the three doxorubicin formulations at different doses and compared to PBS administration. The data are shown in the Supporting Information. The MTD was defined as the maximum dose that led to up to 10% loss of body weight over any 5 day period within the dosing regime, but where body weight returned to baseline levels during the doxorubicin washout period. Using this criterion and consistent with the lower toxicities seen in the rat study, the mice were able to tolerate 2-fold higher doses of D-DOX when compared with L-DOX or DOX. The MTDs (in doxorubicin equivalents) of D-DOX, L-DOX and DOX were 10, 5, and 4.5 mg/kg respectively.

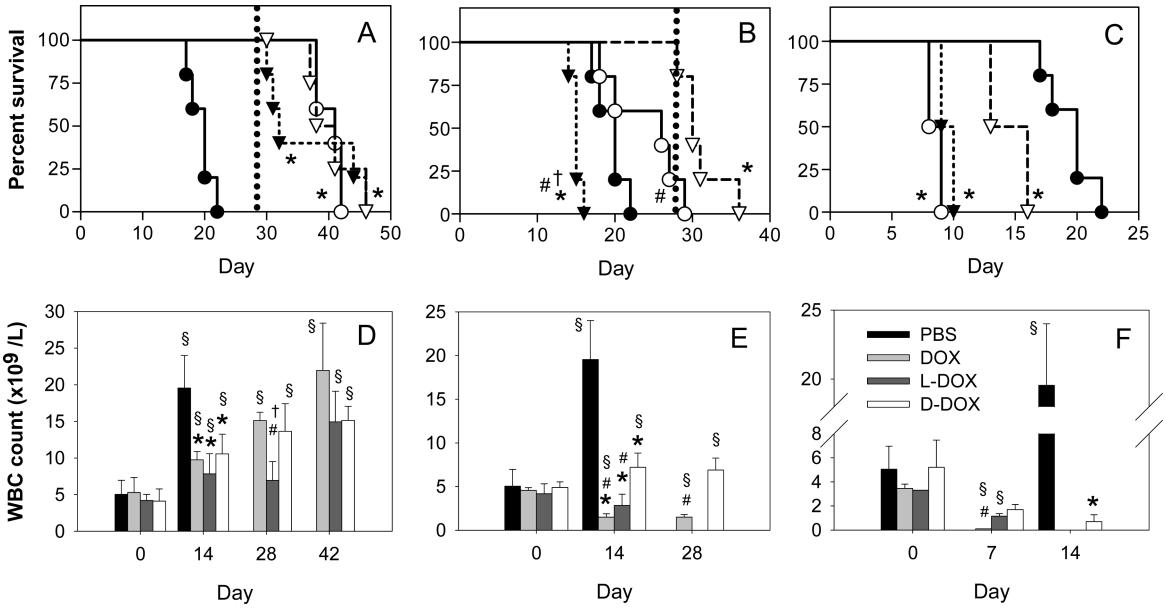

Figure 4. Rat survival curves (upper panels) and peripheral white cell counts (lower panels) as a function of time at 2 mg/kg (A, D), 4 mg/kg (B, E) and 8 mg/kg (C, F) doxorubicin equivalents of PBS (—●—), DOX (—○—), L-DOX (- -▼- -) and D-DOX (– –▽– –). Data represent mean ± SD (n = 5 at the start of dosing). Dotted lines represent the day the final dose was given to the rats. * Indicates significant difference compared to the PBS group; # indicates significant difference compared to the D-DOX group; † indicates significant difference compared to DOX; § indicates significant difference compared to day 0.

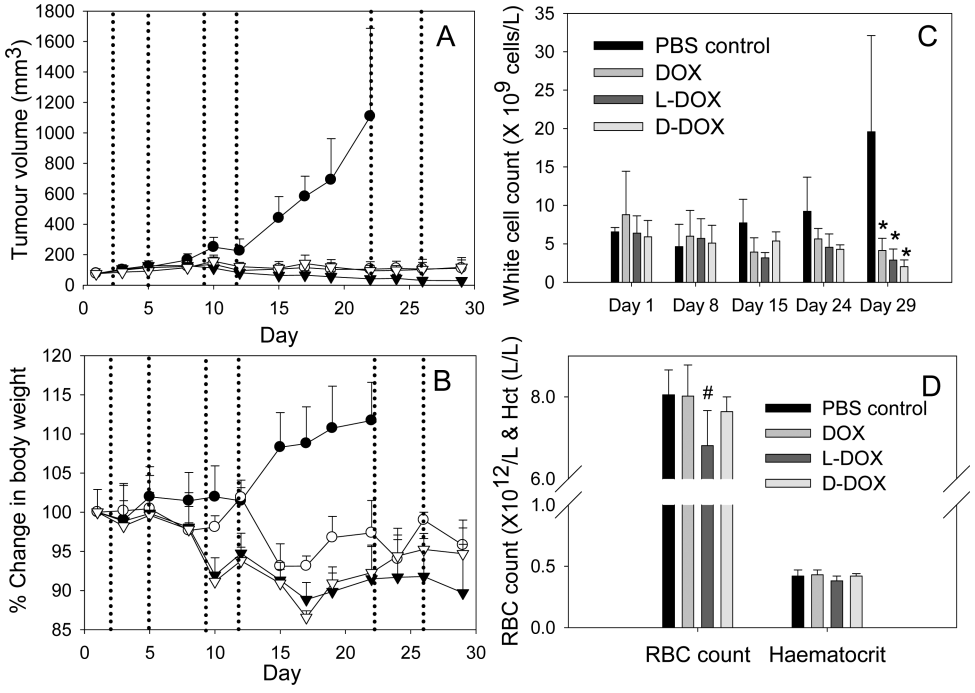

Figure 5. Tumor volume (A), % change in body weight (B), peripheral white cell counts as a function of time (C) and terminal hematocrit (hct) and red blood cell (RBC) counts (D) in MDA MB-231 tumor bearing nu/nu mice administered PBS control (●), DOX (○), L-DOX (▼) or D-DOX (▽) iv at the MTD (4.5 mg/kg DOX, 5 mg/kg L-DOX, 10 mg/kg D-DOX) on day 2, 5, 9, 12, 22 and 26 with a recovery period of 10 days between dosing on day 12 and 22. Dotted lines represent days on which mice were dosed. Data represent mean ± SD (n = 4–7 mice). * indicates significant difference compared to PBS on day 29 although at this point only n = 2 mice remained in the PBS group; # indicates significant difference compared to the DOX group.

In a preliminary activity study using relatively low mouse numbers (n = 4–5), administration of each delivery system at the MTD in the MDA-MB231 tumor xenograft model resulted in significant suppression of tumor growth when compared to control (Supporting Information). Initial indicators of organ toxicity (organ weight changes) were also limited and few differences in liver, lung, heart and kidney weights were evident when compared to animals administered PBS. In all cases, however, doxorubicin administration reduced spleen weight.

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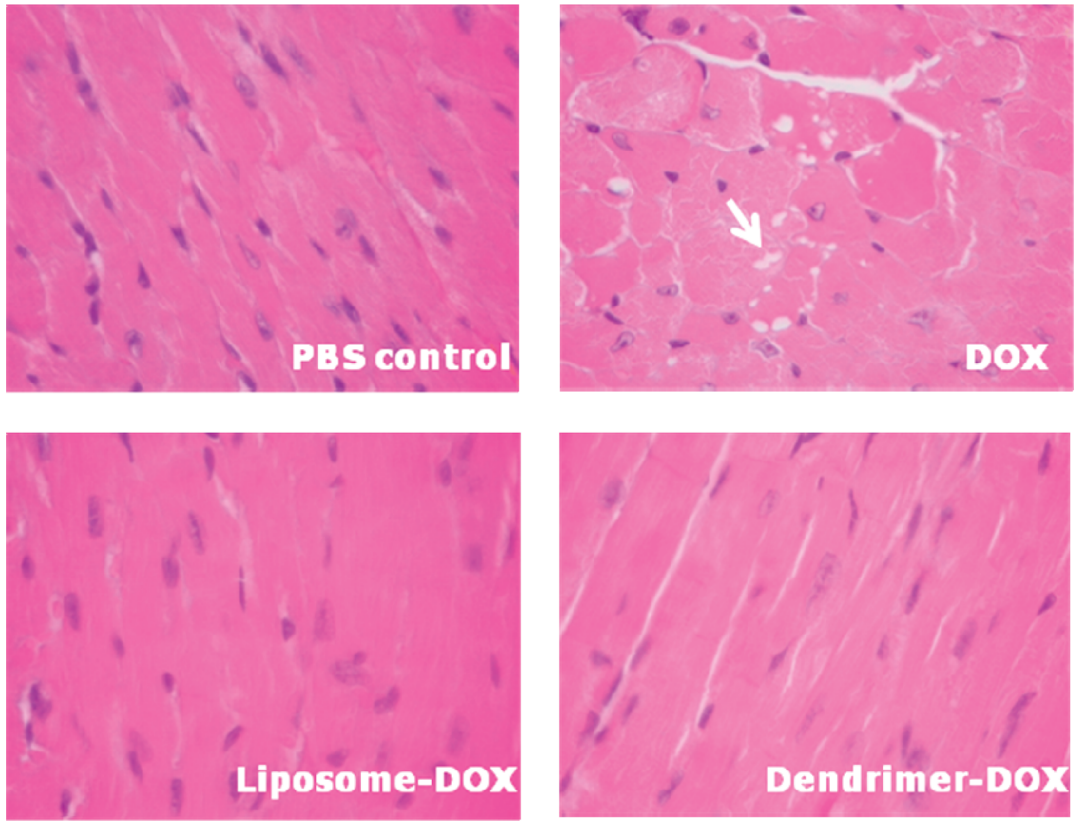

Figure 6. Hematoxylin and eosin stained sections of representative hearts in mice administered PBS control, DOX, L-DOX or D-DOX iv at the MTD twice per week for 2.5 weeks followed by a recovery period of 9 days. Hearts were collected into 10% formalin immediately after death and sectioned at 8 nm thickness. Magnification of heart tissue was at 1000×. Arrow indicates vacuoles in cardiomyocytes.

Since the preliminary xenograft study suggested limited effects on organ weights (with the exception of the spleen), a subsequent study was undertaken to confirm activity using larger numbers of animals and to focus the toxicity profiling end points on known doxorubicin toxicities, namely, hematological end points (WBC, hematocrit, red blood cell count) and cardiotoxicity. At the MTD all dosing regimens resulted in a significant inhibition of the growth of MB-231 tumors (Figure 5A). No significant differences were observed in tumor growth between the 3 doxorubicin groups, suggesting that, at the MTD, the three doxorubicin formulations were equally tumoricidal. Consistent with the MTD studies, animals in each of the doxorubicin groups lost approximately 10% of their initial body weight over the 3 week dosing period. In contrast, mice administered PBS control gained approximately 10% body weight over the dosing period (Figure 5B).

After the dose on day 12, the potential for tumor regrowth was monitored for a further 10 days. Interestingly, in contrast to the rat Walker 256 model, tumor regrowth was not evident in any groups, and in fact tumor size declined after dose cessation such that the terminal tumor volumes were approximately equivalent to those immediately before the first dose. Mice were euthanized at day 29 and markers of hematological toxicity and cardiotoxicity evaluated. Animals administered L-DOX showed a statistically significant (albeit modest) drop in red blood cell counts relative to D-DOX, however hematocrit was not significantly changed and no other significant differences were evident across the groups (Figure 5D). Peripheral white cell counts were suppressed in all groups administered the doxorubicin-containing formulations when compared to the PBS control group and when compared to the white cell counts in the same mice immediately before the first dose (approximately 6−8 × 10^9 cells/L, Figure 5C).

Consistent with the clinical profile of doxorubicin administration, 6 out of 7 mice administered DOX at the MTD displayed histological evidence of cardiotoxicity as evidenced by the loss of cardiac tissue striations, inflammatory cell infiltrates and vacuolization in the cardiomyocytes (Figure 6). In contrast, none of the mice displayed cardiotoxicity in the PBS control group, 2 out of 6 mice displayed cardiotoxicity in the L-DOX group and only 1 out of 7 mice displayed cardiotoxicity in the D-DOX group. Typical histological images of cardiac tissue from each of the 4 groups are shown in Figure 6. Logistic regression analysis revealed that although mice administered D-DOX received more than twice the quantity of doxorubicin as the animals in the DOX group, the incidence of cardiotoxicity in these mice was significantly lower when compared to mice in the DOX group.

■ DISCUSSION

Previous studies have described a G5 doxorubicin-conjugated PEGylated polylysine dendrimer (D-DOX) that shows pharmacokinetic properties consistent with utility as a cytotoxic nanomedicine, namely, low systemic clearance, extended plasma half-life (>30 h) and preferential tumor accumulation.[25,26] D-DOX was designed such that doxorubicin was conjugated to the dendrimer surface via an acid labile hydrazone linker, with the aim of providing specific drug release in an acidic tumor microenvironment and reducing nonspecific release to sites of potential toxicity. Preliminary

evaluation of in vivo activity after 4 × 2 mg/kg doses (doxorubicin equivalents) of D-DOX over a 14 day period revealed significant suppression of tumor growth (using a rat Walker 256 tumor model) compared to control animals administered PBS.[25] An indication of improved toxicity was also seen when compared to administration of the same dose equivalents of a doxorubicin solution formulation or a PEGylated liposome (Caelyx). The current study reports the in vivo activity of D-DOX over more prolonged treatment time scales, and compares activity in a human tumor xenograft in mice to that in the rat Walker 256 tumor model. The current study also evaluates the potential for tumor regrowth after treatment cessation. The rat studies provide a head to head comparison of activity versus toxicity at a series of escalating fixed doses. In contrast, in mice, an indication of maximum potential antitumor activity was provided by dosing at the predetermined maximum tolerated dose (MTD). Toxicity end points (including the MTD), the appearance of PPE and hematological and cardiac toxicity end points are also presented. All data are compared to the clinically available solution of doxorubicin HCl (DOX) and the PEGylated liposomal formulation (L-DOX).

In all groups, and in both rat and mouse xenograft models, administration of the doxorubicin containing formulations led to a significant reduction in tumor growth and the reduction in tumor growth in rat Walker 256 tumors was dose dependent. Consistent with our preliminary studies,[25] and some clinical studies comparing liposomal and simple solution formulations of doxorubicin,[32−34] no significant differences in tumor regression were evident between the D-DOX and L-DOX groups when compared to DOX. This is in contrast to previous studies that have shown considerable differences between PEGylated liposome and dendrimer-based doxorubicin treatments when compared to simple doxorubicin solutions in animal models of drug-resistant cancers.[35−38] The rat and mouse models employed here, however, are doxorubicin sensitive, and differentiating the additional benefit of EPR in the PEGylated constructs may not be possible in systems where DOX treatment alone is highly effective. Nonetheless the data here are consistent with the suggestion that similar activity profiles might be expected for D-DOX when compared to the current industry standard (L-DOX). In spite of the lack of differentiation in tumor regression between D-DOX, L-DOX and DOX, significant differences were evident in activity against Walker 256 rat tumors when compared with the human breast cancer cell line MDA-MB231. Notably, all doxorubicin containing formulations more effectively suppressed tumor growth (and indeed showed some evidence of regression) in the MDA-MB231 tumor model when compared to the Walker rat model at doses that resulted in similar toxicities. Furthermore, when dosed at the MTD for 2 weeks no evidence of tumor regrowth was evident in the MDA-MB231 tumor model regardless of treatment group, whereas in studies with the Walker 256 rat tumors, regrowth was evident as soon as treatment was withdrawn. Interestingly this is the reverse of the relative in vitro cytotoxicities, where in general the Walker cells were more sensitive to the DOX preparation than the MDA-MB231 cells (see Supporting Information). The mice also appeared to tolerate higher doses of the doxorubicin formulations than the rats.

Although few differences between the in vivo cytotoxicity of the three doxorubicin constructs were apparent, differences in toxicity and tolerability were seen and in general D-DOX was less toxic than either L-DOX or DOX. This was evident in a 2-fold increase in MTD, and significantly lower cardiotoxicity in the mouse MDA-MB231 model (in spite of administration at a 2-fold higher dose). Previous biodistribution data in rats suggests similar patterns of biodistribution of total doxorubicin to the heart when administered as D-DOX when compared to DOX and L-DOX.[25] The reduced toxicity of D-DOX may therefore reflect lower nonspecific release at nontarget sites as a result of the acid labile linker, when compared with more nonspecific release in L-DOX. Similar trends were evident in the rat Walker model, where D-DOX resulted in smaller effects on animal growth, and slightly higher terminal spleen weights and WBC counts. Most importantly, the incidence of PPE was significantly lower in animals administered D-DOX when compared to L-DOX. While PPE is commonly associated with swelling of the hands and feet, in this case, the skin toxicity was located primarily on the inner surfaces of the extremities, the neck and the abdomen. The reduced severity and later onset of PPE, and reduced general toxicity and weight loss in the D-DOX groups, especially at the 4 mg/kg dose in rats, allowed larger quantities of doxorubicin to be administered and animals to be maintained in the study for longer time periods. The ability to administer significantly higher total quantities of doxorubicin using the D-DOX construct resulted in an alternate toxicity being observed. Toward the end of the final dosing period, approximately half of the rats in the 4 mg/kg D-DOX group displayed reduced motor coordination. This effect has been observed previously after administration of high doses of doxorubicin and is a result of drug activity in the dorsal root ganglion cells of the peripheral nervous system.[28−31] This effect was not seen in the DOX or L-DOX groups, as animals in these groups generally required euthanasia well before the onset of this symptom.

In summary, this study has demonstrated that conjugation of doxorubicin via an acid labile linker to a PEGylated polylysine dendrimer provides equivalent chemotherapeutic efficacy (in the models studied) when compared to the administration of a simple solution formulation of the drug (DOX) and a PEGylated liposomal formulation (L-DOX). In addition, the dendrimer formulation showed improved toxicity end points in rodents including reduced PPE and cardiotoxicity, two of the major side effect limitations of current doxorubicin therapies.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional experimental details as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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