

# A single dose of doxorubicin-functionalized bow-tie dendrimer cures mice bearing C-26 colon carcinomas

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The antitumor effect of doxorubicin (DOX) conjugated to a biodegradable dendrimer was evaluated in mice bearing C-26 colon carcinomas. An asymmetric biodegradable polyester dendrimer containing 8–10 wt % DOX was prepared. The design of the dendrimer carrier optimized blood circulation time through size and molecular architecture, drug loading through multiple attachment sites, solubility through PEGylation, and drug release through the use of pH-sensitive hydrazone linkages. In culture, dendrimer–DOX was >10 times less toxic than free DOX toward C-26 colon carcinoma cells after exposure for 72 h. Upon i.v. administration to BALB/c mice with s.c. C-26 tumors, dendrimer–DOX was eliminated from the serum with a half-life of  $16 \pm 1$  h, and its tumor uptake was ninefold higher than i.v. administered free DOX at 48 h. In efficacy studies performed with BALB/c mice bearing s.c. C-26 tumors, a single i.v. injection of dendrimer–DOX at 20 mg/kg DOX equivalents 8 days after tumor implantation caused complete tumor regression and 100% survival of the mice over the 60-day experiment. No cures were achieved in tumor-implanted mice treated with free DOX at its maximum tolerated dose (6 mg/kg), drug-free dendrimer, or dendrimer–DOX in which the DOX was attached by means of a stable carbamate bond. The antitumor effect of dendrimer–DOX was similar to that of an equimolar dose of liposomal DOX (Doxil). The remarkable antitumor activity of dendrimer–DOX results from the ability of the dendrimer to favorably modulate the pharmacokinetics of attached DOX.

antitumor | molecular architecture | therapeutic effect | nanomedicine | dendrimer prodrug

High-molecular-weight (MW) water-soluble polymers are being extensively investigated for use as drug carriers (1). Incorporating low-MW drugs into high-MW polymeric systems can increase the drug circulation time, because the rate of renal filtration is related to the hydrodynamic volume of a solute, with larger molecules being eliminated more slowly (1–3). Attachment of anticancer drugs to polymers can improve their passive targeting to tumors because of the increased permeability of tumor vasculature to macromolecules (4, 5) and the decreased lymphatic drainage from the tumor, a phenomenon known as the enhanced permeation and retention (EPR) effect (1, 6). Passive targeting decreases the systemic toxicity and enhances the therapeutic efficacy of conjugated drugs (7, 8).

For polymers, tumor accumulation generally increases with MW, but urinary clearance decreases with MW; therefore, polymers used clinically must be biodegradable to achieve good tumor targeting while preventing undesired long-term tissue accumulation. A low polydispersity for the polymer is also important, because polymers with the same average MWs but different MW distributions can have different biodistribution profiles because of the presence of species with vastly different sizes within broad polydispersity samples (9). Thus, the ability to prepare biodegradable, high-MW polymers with narrow polydispersities is of the utmost importance for achieving favorable tumor accumulation of attached drugs.

Relative to linear polymers, dendritic polymers possess unique features that may be advantageous for drug delivery (10, 11). Dendrimers are highly branched and contain a well defined number of peripheral groups that can be functionalized with drugs, targeting moieties, or solubilizing groups in controlled ratios. The stepwise synthesis of dendrimers provides macromolecules with a unique MW or very narrow polydispersity. Moreover, the branched structures of dendrimers may also impede their passage through small pores, such as those of the glomerular filtration barrier, and, thus, a dendrimer may be eliminated more slowly than a linear polymer with the same molar mass (12–14). Despite these significant potential benefits, few *in vivo* antitumor evaluations of dendrimer-based drug carriers have been reported (15, 16), possibly because dendrimers that are large enough to exhibit long circulation times (so that they can exploit EPR effect-mediated tumor targeting) (>5 nm) can be difficult to prepare, with low yields resulting from multistep syntheses. In addition, the attachment of drugs at the periphery of a dendrimer can lead to aggregation (15), resulting in a polydisperse material.

By combining a monodisperse dendrimer with narrow polydispersity poly(ethylene oxide) (PEO, also referred to as PEG) (13, 17–21), one can rapidly increase the hydrodynamic size of a dendrimer and maintain good size homogeneity while at the same time increasing the normally low drug-loading capacity of linear PEO (22). In addition, because PEO is known to be an effective steric stabilizer (3), the aggregation sometimes associated with drug-functionalized dendrimers should be reduced (15). Through careful design and synthesis, we have prepared polyester dendrimer–PEO hybrids that exhibit high water solubility, tunable MWs, tunable drug-loading capacities, biodegradability, low polydispersity, low toxicity, and favorable pharmacokinetic profiles in tumor-implanted mice (13, 20). Polymeric carriers possessing all of these features are rare. Here, we show that a single injection of a high-MW dendrimer–PEO–doxorubicin conjugate substantially inhibits the progression of the doxorubicin (DOX)-insensitive C-26 tumor and even provides a cure at certain doses. The results suggest that dendrimer–PEO hybrids are promising carriers of anticancer therapeutics for the treatment of solid tumors.

## Results and Discussion

**Design of a PEO–Dendrimer Hybrid as a Drug Carrier.** In recent years, our group has reported on the synthesis and biological evaluation

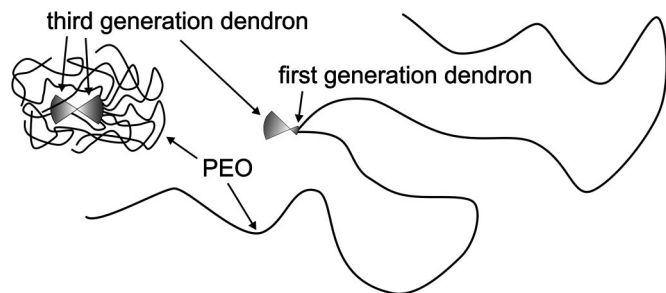
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Abbreviations: MW, molecular weight; EPR, enhanced permeation and retention; DOX, doxorubicin; PEO, poly(ethylene oxide).

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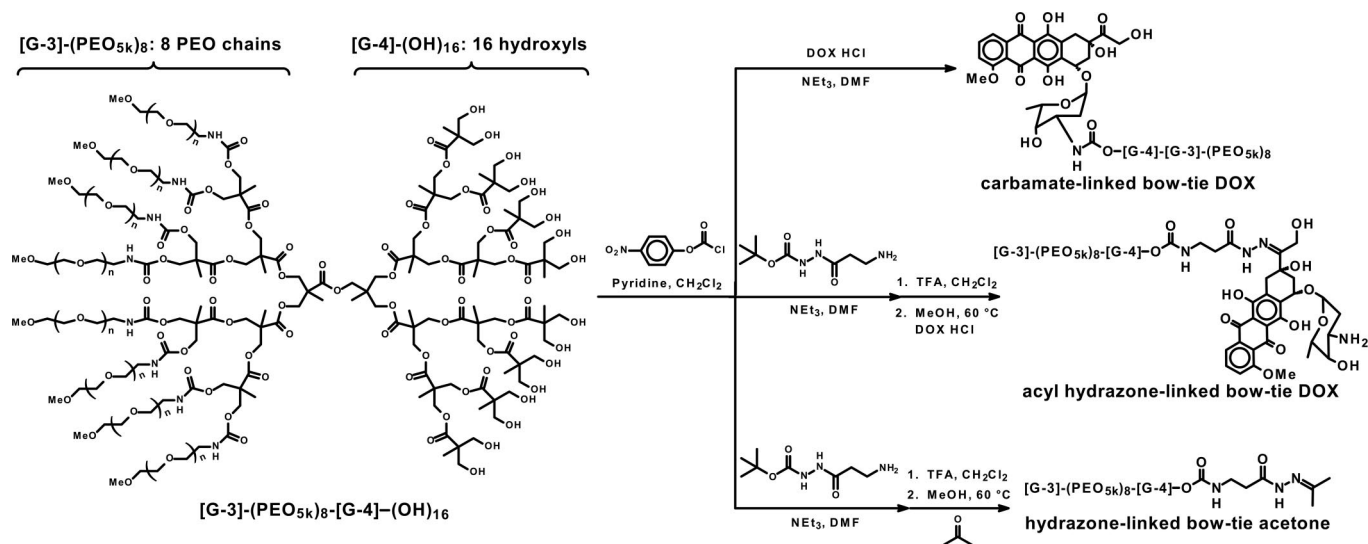


**Fig. 1.** Cartoon representation of two bow-tie dendrimers with the same mass of PEO attached (40,000 Da). The more compact [G-3]-(PEO<sub>5k</sub>)<sub>8</sub>-[G-3]-(OH)<sub>8</sub> dendrimer on the left is composed of eight 5,000-Da PEO chains attached to one side of the dendrimer; the less branched [G-1]-(PEO<sub>20k</sub>)<sub>2</sub>-[G-3]-(OH)<sub>8</sub> dendrimer on the right is composed of two 20,000-Da PEO chains attached to one side of the dendrimer. The other half of each dendrimer is used for drug attachment and is presumably wrapped and shielded by the PEO chains to some extent.

of biodegradable polyester dendrimers based on 2,2-bis(hydroxymethyl)propionic acid and their hybrids with PEO (13, 17–21). *In vitro* and *in vivo* studies have shown that, in contrast to some poly(amidoamine) dendrimers (23), the polyester dendrimer scaffold is hydrolytically degradable and less toxic and does not accumulate in vital organs (13, 19). We recently reported the synthesis of nanometer-sized asymmetric polyester dendrimers in which the peripheral hydroxyl groups of one hemisphere of the dendrimer are functionalized with PEO chains and the peripheral hydroxyl groups on the opposite hemisphere are left unfunctionalized for the subsequent attachment of a drug or reporter payload (Figs. 1 and 2). We designate dendrimers with this molecular architecture as “bow-tie” dendrimers. The number and length of the PEO chains and the number of drug attachment sites can be varied, allowing access to carriers with different sizes, architectures (more or less branched), and drug-loading capacities. In mice, bow-tie dendrimers with molecular masses of  $>\approx 40$  kDa exhibit plasma elimination half-lives in excess of 24 h (13), and, because a long blood circulation time is a prerequisite for tumor targeting using the EPR effect (1, 6), bow ties with molecular masses  $>40$  kDa are acceptable candidates for passive tumor targeting.

It is important to note that, in previous work, we have found that the number of PEO arms attached to the bow tie can have a significant influence on its biodistribution. For example, a radioiodinated [G-3]-(PEO<sub>5k</sub>)<sub>8</sub>-[G-3]-(OH)<sub>8</sub> bow tie (molecular mass of  $\approx 45$  kDa) had a plasma elimination half-life of 31 h, whereas a radioiodinated [G-1]-(PEO<sub>20k</sub>)<sub>8</sub>-[G-3]-(OH)<sub>8</sub> bow tie (molecular mass of  $\approx 44$  kDa) had a plasma elimination half-life of  $<2$  h due to rapid uptake by the liver (13). Here, “[G-3]-(PEO<sub>5k</sub>)<sub>8</sub>” designates a third-generation dendron with eight peripheral attachment sites for PEO chains (each with a number average molecular mass of 5,000 Da), whereas “[G-3]-(OH)<sub>8</sub>” designates an attached third-generation dendron with eight hydroxyls available for conjugation (Fig. 1). The radioiodinated phenols used for these biodistribution experiments were located on the periphery of the dendron opposite to the dendron with the attached PEO chains. Because polymers containing iodinated phenols are known to exhibit high levels of liver uptake (19, 24), these results indicate that more branched bow ties offer better steric protection of molecules located on the periphery of the opposite dendron (Fig. 1). The ability of the PEO chains to sterically shield the payload of a bow tie from its external environment should be useful when applied to the delivery of enzymatically sensitive or hydrophobic drugs.

**Synthesis of a Bow-Tie Polymer–DOX Conjugate.** We chose to use a [G-3]-(PEO<sub>5k</sub>)<sub>8</sub>-[G-4]-(OH)<sub>16</sub> bow tie with a molecular mass of 45 kDa for this work (Fig. 2). Although other bow ties with molecular masses of  $>40$  kDa have suitable circulation half-lives for passive tumor targeting ( $t_{1/2}$  for [G-2]-(PEO<sub>10k</sub>)<sub>4</sub>-[G-3]-(OH)<sub>8</sub>, [G-2]-(PEO<sub>20k</sub>)<sub>4</sub>-[G-3]-(OH)<sub>8</sub>, [G-3]-(PEO<sub>10k</sub>)<sub>8</sub>-[G-3]-(OH)<sub>8</sub>, and [G-3]-(PEO<sub>20k</sub>)<sub>8</sub>-[G-3]-(OH)<sub>8</sub> is 26, 25, 40, and 50 h, respectively), we chose the [G-3]-(PEO<sub>5k</sub>)<sub>8</sub>-[G-4]-(OH)<sub>16</sub> bow tie because it is more branched than the [G-2]-(PEO<sub>10/20k</sub>)<sub>4</sub> bow ties and contains less PEO per dendron (by weight) than the [G-3]-(PEO<sub>10/20k</sub>)<sub>8</sub> bow ties. Highly branched bow ties exhibit good steric protection of their payloads, whereas bow ties containing just enough PEO to prevent renal clearance have higher theoretical drug-loading capacities (on a weight percent basis). We had previously reported (20) a [G-3]-(PEO<sub>5k</sub>)<sub>8</sub>-[G-3]-(OH)<sub>8</sub> bow-tie dendrimer with only eight drug attachment sites, but this dendrimer had a relatively low drug-carrying capacity; the yield of hydrazone formation with polyester den-



**Fig. 2.** Functionalization of the [G-3]-(PEO<sub>5k</sub>)<sub>8</sub>-[G-4]-(OH)<sub>16</sub> bow-tie dendrimers for therapeutic studies. DOX is linked to the bow tie by means of a carbamate (top) or acyl hydrazone (middle) linkage. In the bottom route, hydrazide groups of the bow tie are blocked upon reaction with acetone. The top and bottom bow ties represent control treatments.

dimer systems is  $\approx 50\%$  (19, 21). Therefore, to achieve a drug loading comparable to polymers or liposomes that have been previously used to deliver DOX ( $\approx 10$  wt %) (7, 25), the drug-carrying capacity of the bow tie was doubled by increasing the generation of the drug-carrying dendron from [G-3] to [G-4].

DOX is an ideal drug to deliver because it is potent, and its therapeutic efficacy in a variety of *in vivo* tumor models and in humans is well documented. Several groups have described the preparation and evaluation of polymer conjugates with DOX (7, 26–29). Most extensively investigated for the delivery of DOX is the linear polymer *N*-(2-hydroxypropyl)methacrylamide, which has shown promising results in animal models (7) and clinical trials (30, 31). Additionally, a clinically approved liposomal drug-delivery system for DOX exists (Doxil), which provides a commercially available positive control with which to assess the delivery potential of our bow-tie system. A pH-sensitive acyl hydrazone linkage was chosen as the method of drug attachment, because the drug can be selectively released either in the mildly acidic extracellular environment of a tumor (32) or upon pinocytotic uptake and trafficking into acidic endosomal or lysosomal subcellular compartments (33, 34).

Coupling of hydrazide linkers to the hydroxyl groups of the bow tie, followed by hydrazone formation with DOX hydrochloride and subsequent chromatographic separation from free DOX, provided the dendrimer–DOX conjugate (Fig. 2). DOX loading, which was quantified by using UV-visible spectroscopy, was consistently found to be 8–10 wt % for different batches. Notably, the bow-tie DOX conjugate was readily dissolved in water at DOX concentrations as high as 6 mg/ml ( $\approx 60$  mg/ml polymer), indicating that the PEO arms of the bow-tie dendrimer can shield the hydrophobic drug moieties at the core of the molecule, perhaps in a structure similar to that of a unimolecular micelle. A volume average hydrodynamic diameter of 8 nm for the conjugate was determined by using dynamic light scattering, indicating that intermolecular aggregation did not occur.

Control bow-tie dendrimers containing only the hydrazide linker (blocked with acetone) or with DOX attached by means of a more stable carbamate linkage were also synthesized (Fig. 2).

To confirm that DOX was released from the hydrazone-linked dendrimer–DOX conjugate in a pH-dependent manner, DOX release was monitored chromatographically at pH 7.4 and 5.0 (Fig. 4, which is published as supporting information on the PNAS web site). Drug was released from the dendrimer rapidly at pH 5.0, reaching 100% release within 48 h ( $t_{1/2} = 6 \pm 1$  h). As desired, only a small amount of drug ( $<10\%$ ) was released at pH 7.4 over the same period.

**Cytotoxicity in Cell Culture.** After a 72-h incubation period, the dendrimer–DOX conjugate was found to be considerably less toxic toward cultured C-26 cells than free DOX on an equimolar basis ( $IC_{50, DOX} = 0.08 \pm 0.02$   $\mu\text{g/ml}$ ;  $IC_{50, \text{hydrazone bow-tie DOX}} = 1.4 \pm 0.2$   $\mu\text{g/ml}$ ). The bow tie with DOX linked by means of the stable carbamate linkage had an  $IC_{50}$  of  $2.0 \pm 0.2$   $\mu\text{g/ml}$ . The lesser cytotoxic activity of the polymer DOX preparations is presumably due to the slower rate of cellular uptake for the dendrimers when compared with the free drug (15) and to the gradual release of free drug from the polymers due to hydrolysis of the linkers and the polyester dendrimer backbone (13).

**Biodistribution Studies in Tumor-Implanted Mice.** Biodistribution experiments in BALB/c mice bearing s.c. C-26 tumors were performed with the hydrazone-linked bow-tie DOX conjugate to confirm that its pharmacokinetic behavior was similar to that of the bow tie without attached drug (13). Serum concentrations of DOX (measured by DOX fluorescence) decreased over time in a log-linear manner from 2 to 48 h and had an elimination half-life of  $16 \pm 1$  h (Fig. 5, which is published as supporting

information on the PNAS web site). The bow tie without drug attached displayed two-phase distribution kinetics, with a blood elimination half-life of  $31 \pm 2$  h (13). The long circulation half-life of dendrimer–DOX conjugates contrasts with the short half-life of the free drug, which is  $<10$  min (35). The tumor concentrations of DOX measured 48 h after administration of either dendrimer–DOX (20 mg/kg DOX) or free DOX (6 mg/kg) were approximately nine times higher for mice treated with dendrimer–DOX on a percent injected dose per gram of tumor basis. The enhanced tumor uptake of the dendrimer-bound drug is a reflection of its longer circulation half-life, which exploits passive targeting by means of the EPR effect.

**Maximum Tolerated Dose of Hydrazone-Linked Bow-Tie DOX in Healthy Mice.** To determine the *in vivo* maximum tolerated dose for bow-tie DOX, the conjugate was administered i.v. to BALB/c mice at doses of 0 (PBS), 20, 40, and 60 mg/kg DOX equivalents. The weights and general health of the mice were monitored until the ninth day after injection (Fig. 6, which is published as supporting information on the PNAS web site), when one mouse in the group receiving the highest dose was lethargic and showed obvious signs of morbidity. The other two mice in this group also exhibited signs of toxicity (reduced weight and ruffled fur), whereas mice in the groups receiving lower doses of dendrimer appeared healthy. Mice receiving 20 mg/kg DOX equivalents showed no weight loss over the duration of the experiment; however, they did not gain weight at the same rate as control mice (which received PBS). Mice that were given 40 mg/kg DOX equivalents lost weight during the study, but not  $>10\%$  of their initial mass. Mice receiving 60 mg/kg DOX equivalents lost  $>15\%$  of their body weight.

At day 9, all of the mice were killed; blood was then collected, and the serum was separated and analyzed. There was a significant increase in the serum creatine kinase, lactic dehydrogenase, and serum transaminase values in animals that received the 40 and 60 mg/kg doses compared with animals that received saline or the 20 mg/kg dose, indicating the presence of damage to muscle tissue and to the liver at these dose levels. Thus, we conclude that the maximum tolerated single dose is between 20 and 40 mg/kg DOX equivalents or between  $\approx 200$  and 500 mg/kg dendrimer–DOX in healthy BALB/c mice. The maximum tolerated dose for a single injection of the hydrazone-linked dendrimer–DOX is similar in the BALB/c mouse strain to DOX delivered in liposomes (36).

**Chemotherapy Experiments.** C-26 colon carcinoma was chosen as the tumor model in which to test the bow-tie DOX because it represents a challenging cancer cell line that is relatively sensitive to free DOX in cell culture but not *in vivo*, a finding that has been attributed to the inability of the drug to attain sufficient intratumor concentrations (37). Therapeutic success with this model has been achieved with liposomal anthracyclines and is accredited to EPR effect-mediated tumor targeting (25, 38). Because dendrimer–DOX provides higher intratumor levels of DOX than the free drug, we reasoned that the therapeutic efficacy of dendrimer–DOX would exceed that of the free drug.

To determine the optimal dosing schedule for antitumor therapy, BALB/c mice bearing s.c. C-26 tumors were administered a single dose of dendrimer–DOX (10 mg/kg DOX) on various days after tumor inoculation. Five different groups of mice were treated with a single i.v. injection of polymer on day 2, 4, 8, 12, or 16 after their tumors were implanted. The effect of dosing date on therapeutic efficacy is clearly illustrated in plots of mouse survival versus time (Table 1; see also Fig. 7, which is published as supporting information on the PNAS web site). The survival times of mice dosed on days 4, 12, or 16 were not statistically different by using the log-rank test ( $P > 0.4$ ). Mice treated on day 2 fared slightly better, and in comparison

**Table 1. Therapeutic efficacy of various DOX and control formulations for the treatment of s.c. C-26 colon carcinomas in BALB/c mice**

| Experiment                               | Treatment details                | No. of mice per group | DOX, mg/kg | Median survival time, days* | Significance, P |
|--|----------------------------------|-----------------------|------------|-----------------------------|-----------------|
| Dosing schedule experiment <sup>†</sup>  | Day 2                            | 10                    | 10         | 30.5 (1/10)                 | 0.035           |
|  | Day 4                            | 10                    | 10         | 24 (0/10)                   | NS              |
|  | Day 8                            | 10                    | 10         | 38.5 (1/10)                 | 0.004           |
|  | Day 12                           | 10                    | 10         | 22 (0/10)                   | NS              |
|  | Day 16                           | 10                    | 10         | 24 (0/10)                   | —               |
| Dose–response experiment <sup>†</sup>    | PBS                              | 10                    | 0          | 22 (0/10)                   | —               |
|  | DOX HCl                          | 10                    | 6          | 24 (0/10)                   | NS              |
|  | Hydrazone-linked bow-tie DOX     | 10                    | 1          | 24 (0/10)                   | NS              |
|  | Hydrazone-linked bow-tie DOX     | 10                    | 3          | 22 (2/10)                   | NS              |
|  | Hydrazone-linked bow-tie DOX     | 10                    | 6          | 35.5 (2/10)                 | 0.016           |
|  | Hydrazone-linked bow-tie DOX     | 10                    | 20         | 60 (10/10)                  | <0.0001         |
|  | Doxil                            | 10                    | 6          | 39 (3/10)                   | 0.0001          |
|  | Doxil                            | 10                    | 20         | 60 (9/10)                   | 0.0001          |
| Control polymers experiment <sup>†</sup> | PBS                              | 7                     | 0          | 28 (0/7)                    | —               |
|  | Hydrazone-linked bow-tie acetone | 8                     | 0          | 34 (0/8)                    | 0.051           |
|  | Carbamate-linked bow-tie DOX     | 10                    | 20         | 30 (0/10)                   | 0.019           |

P values are calculated relative to PBS treatment, except in the dosing schedule experiment, where P values are calculated relative to treatment on day 16. NS, not significant.

\*Numbers in parentheses refer to the number of tumor-free mice alive at the end of the study.

<sup>†</sup>Single dose of hydrazone-linked bow-tie DOX on specified day after tumor implantation.

<sup>†</sup>Single dose of specified treatment on day 8 after tumor implantation.

with mice treated on days 4, 12, and 16, the differences in survival were more significant ( $P = 0.09, 0.03, \text{ and } 0.035$ , respectively). Mice treated on day 8 responded the most favorably to treatment, a result that was statistically different from the mice treated on days 4, 12, and 16 ( $P = 0.02, 0.01, \text{ and } 0.004$ , respectively).

The dendrimer–DOX, like many other polymer- and liposome-delivered drugs, is designed to exploit the EPR phenomenon (1, 6, 8). The best response was achieved by dosing on day 8, probably because the tumor vasculature was developed sufficiently to allow targeting by the EPR effect (25). Tumors that were treated too early likely had not developed the proper vasculature for effective tumor targeting, and by the time the vasculature had developed more fully, the dendrimer and drug had been eliminated. Tumors that were treated too late had progressed to a size that was too large to be affected by the quantities of drug administered, despite EPR effect targeting. This result is similar to what is observed when mice implanted with the C-26 carcinoma are treated with a sterically stabilized liposome DOX preparation (25).

A dose–response experiment was performed by monitoring tumor growth and survival of BALB/c mice treated with a single dose of dendrimer–DOX 8 days after implantation of a s.c. C-26 tumor. A dependence of tumor growth rate and survival on dosage was observed for the dendrimer drug (Table 1 and Fig. 3a; see also Fig. 8a, which is published as supporting information on the PNAS web site). Remarkably, at the highest dose administered (20 mg/kg DOX equivalents), complete tumor regression was observed, resulting in 100% survival of mice in this treatment group ( $n = 10$ ) over the 60-day experiment.

As a positive control in this experiment, Doxil was concurrently tested at the two highest DOX concentrations (Table 1 and Figs. 3b and 8b). At the highest administered dose (20 mg/kg DOX), the efficacy of Doxil was nearly identical to that of dendrimer–DOX when comparing the tumor growth rates and survival curves for mice receiving the two treatments.

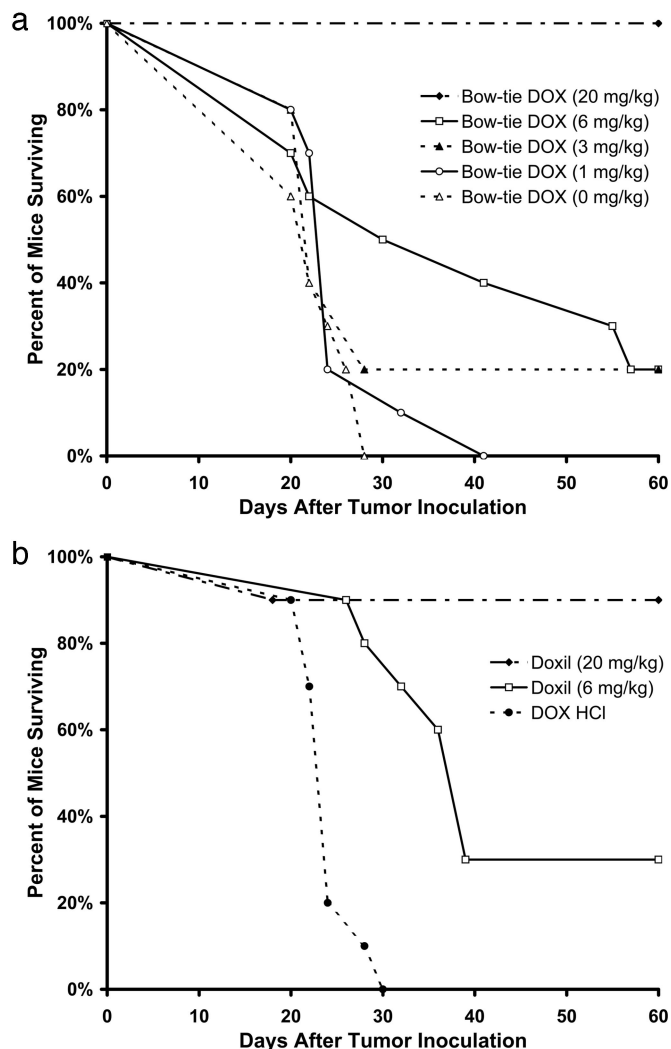
However, with regard to toxicity as detected by the loss of body weight, the mice receiving dendrimer–DOX fared better, with toxicity being most noticeable when Doxil was administered at 20 mg/kg DOX (Fig. 9, which is published as supporting

information on the PNAS web site). Mice receiving Doxil at this concentration exhibited near-toxic levels of weight loss, with the mean weight loss at nadir (day 16) being 12.2% for Doxil and 7.9% for dendrimer–DOX ( $P = 0.05$ ). Although weight loss was apparent in mice given dendrimer–DOX at the same concentration, the levels of weight loss were less severe throughout the study, and their weights returned to their initial values within 2 weeks of treatment.

In contrast to the hydrazone-linked dendrimer–DOX conjugate and the liposome-based delivery vehicle, no cures were achieved in mice treated with free DOX near the maximum tolerated dose of the drug (6 mg/kg; Table 1 and Figs. 3b and 8b). In a separate experiment, dendrimer lacking the attached DOX (238 mg of polymer/kg; Fig. 2) or dendrimer with DOX attached by means of stable carbamate linkages (20 mg/kg DOX equivalents; Fig. 2) was i.v. administered to tumor-implanted mice 8 days after tumor implantation (Table 1; see also Fig. 10, which is published as supporting information on the PNAS web site). Unlike Schätzlein and coworkers (39), who observed potent antitumor activity for cationic dendrimers without a need for attached drugs, we observed only very weak antitumor activity for our drug-free dendrimers. Thus, the maximum therapeutic effect of dendrimer–DOX required the presence of drug on the polymer, long circulation of the carrier, and an appropriate rate of drug release from the bow tie.

Additional study will be required to establish the precise *in vivo* mechanism of action for dendrimer–DOX. For example, it would be of interest to determine whether DOX is released within the cell or outside the cell, because the route of cellular uptake for a drug dictates whether drug resistance pathways can be avoided. Furthermore, chemotherapy studies where treatment commences on later days (possibly consisting of multiple doses) would be necessary to determine whether dendrimer–DOX also behaves as a potent antitumor agent when acting on larger tumors.

The success of the bow-tie dendrimer in this DOX-insensitive tumor model is remarkable in light of previous work toward the same goal. Polydisperse linear polymer DOX conjugates achieved some success in slowing the growth of s.c. C-26 tumors



**Fig. 3.** Survival versus time for BALB/c mice bearing s.c. C-26 tumors. In *a*, treatment consisted of a single i.v. dose of hydrazone-linked bow-tie DOX given 8 days after tumor implantation. In *b*, treatment consisted of a single i.v. injection of either free DOX or Doxil given 8 days after tumor implantation. Doses (in DOX equivalents) are specified in the key. ( $n = 10$  for each group.)

(single doses of 5–20 mg/kg DOX equivalents), but no cures were reported (40, 41). Significant C-26 tumor growth inhibition and some cures were attained in mice treated with DOX encapsulated in block copolymer micelles (42–44), but in all cases, multiple doses of carrier were required, and in one case, therapeutic activity significantly different from free drug was achieved only when each dose was >100 mg/kg DOX equivalents (42). To date, the most effective DOX carriers for treatment of this tumor model have been sterically stabilized liposomes. Most impressive was the finding by Huang *et al.* (25) that treatment with three weekly doses of liposomal DOX (6–10 mg/kg DOX per dose) starting on day 10 resulted in complete tumor regression and 100% cures. Single doses of liposomal DOX (10 mg/kg DOX) resulted in slower tumor growth and some cures, provided that treatment commenced on or before day 9.

An important observation of the present work is that the dendrimer–DOX is as effective as the clinically approved drug carrier Doxil in a DOX-insensitive solid tumor. Although the efficacies of the two DOX delivery systems examined here were similar at similar doses, bow-tie dendrimers possess certain

advantages that make them potentially more versatile than liposomes or other drug carriers. First, in contrast to liposomes (3, 45) and micelles (46), the covalent nature of the drug incorporation in the bow-tie system should allow for the facile attachment of a wide variety of drugs, regardless of their hydrophobicity and charge. Second, because the bow tie is assembled covalently, the material can be stored as a solid and is easily rehydrated without the need for solubilizing excipients. The resulting solid pharmaceutical would be more stable, more easily stored, and more easily formulated. Finally, bow-tie drug conjugates are topologically similar to liposomes in that their shapes are thought to be globular, with the drug located near their cores. Thus, although it is known that the backbone hydrophobicity and charge can significantly influence the solubility and biodistribution profiles of linear polymers (47), on the contrary, one would expect the distribution properties of bow-tie drug conjugates to be similar for different classes of drugs because of a lack of solvent accessibility near the dendrimer core.

The activity of the dendrimer–DOX conjugate *in vivo*, despite its reduced *in vitro* toxicity relative to free DOX, is convincing evidence of the dendrimer's ability to modulate the pharmacokinetic profiles of attached anticancer drugs. Furthermore, the observation that the antitumor efficacy of dendrimer–DOX compares favorably with the FDA-approved Doxil leads us to believe that bow-tie dendrimers, or similar dendritic polymers, are promising carriers of anticancer therapeutics. Although much work needs to be performed to demonstrate that bow ties are general drug carriers, the possibility that a single carrier can be used to impart multiple classes of drugs, imaging agents, or combinations of agents with the same solubilities, biodistribution, and pharmacokinetic profiles warrants further investigation of these versatile molecules.

### Materials and Methods

Detailed descriptions of the synthesis of the bow-tie conjugates, *in vitro* DOX release studies, cell toxicity assays, and biodistribution studies in tumor-implanted mice are described in detail in *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site.

**Animals and Tumor Models.** All animal experiments were performed in compliance with National Institutes of Health guidelines for animal research under a protocol approved by the Committee on Animal Research at University of California (San Francisco, CA) (UCSF). C-26 colon carcinoma cells obtained from the UCSF cell culture facility were cultured in RPMI medium 1640 containing 10% FBS. For biodistribution and chemotherapy studies, 4- to 8-week-old female BALB/c mice were inoculated s.c. under anesthesia with  $3\text{--}4 \times 10^5$  tumor cells in a volume of 50  $\mu\text{l}$  injected directly into the shaved right flank. While still under anesthesia, the mice were randomized and coded by ear punching.

### Maximum Tolerated Hydrazone-Linked Bow-Tie DOX Conjugate i.v. Dose Studies.

Four-week-old female BALB/c mice (three per group) were administered by i.v. injection the hydrazone-linked bow-tie DOX conjugate in  $\approx 200 \mu\text{l}$  of PBS at concentrations of 20, 40, and 60 mg/kg DOX (220, 430, and 650 mg/kg conjugate, respectively). The mice were weighed and monitored daily until 9 days after injection, when one of the mice in the 60 mg/kg group was found near death; at this point, all of the groups were euthanized. Blood was collected by heart puncture, and the serum was separated, frozen at  $-20^\circ\text{C}$ , and submitted to the Comparative Pathology Laboratory (University of California, Davis, CA) for blood chemistry analysis.

**Chemotherapy Experiments.** Mice were inoculated with C-26 tumors as described above and were then randomized with 10 mice

per group and numbered. Mice were weighed, and tumor sizes were monitored daily during the experimental period. The tumor volume was estimated by measuring three orthogonal diameters ( $a$ ,  $b$ , and  $c$ ) with calipers; the volume was calculated as  $(a \times b \times c) \times 0.5 \text{ cm}^3$ . Tumors that were just palpable were defined as  $1 \times 1 \times 1 \text{ mm}$ . In each experiment, the mice were monitored for up to 60 days after inoculation or until one of the following conditions for euthanasia was met: (i) the mouse's body weight dropped below 15% of its initial weight, (ii) the mouse's tumor was  $>2.0 \text{ cm}$  across in any dimension, (iii) the mouse became lethargic or sick and unable to feed, or (iv) the mouse was found dead. On day 60, all surviving mice were euthanized; however, if any of the surviving mice had palpable tumors on day 60, monitoring of all mice remaining in the experiment continued until day 90, at which point the mice were euthanized. Survival analysis by using the log-rank test was performed by using MedCalc 8.2.1.0 for Windows (MedCalc Software, Mariakerke, Belgium).  $P < 0.05$  was considered significant.

In the experiment intended to determine the optimal dosing date for antitumor therapy, mice were administered by tail vein injection a single dose of hydrazone-linked bow-tie DOX conjugate in  $\approx 200 \mu\text{l}$  of PBS (10 mg/kg DOX) on various days after tumor inoculation. Five different groups of mice were treated

with a single i.v. injection of polymer on day 2, 4, 8, 12, or 16 after tumor implantation.

In the experiments intended to determine the effect of dosage on efficacy, 8 days after tumor implantation, mice were administered by tail vein injection a single dose of hydrazone-linked bow-tie DOX conjugate in  $\approx 200 \mu\text{l}$  of PBS at concentrations of 1, 3, 6, and 20 mg/kg DOX. Control groups were treated with Doxil (6 and 20 mg/kg DOX in  $\approx 200 \mu\text{l}$  of PBS), DOX HCl (6 mg/kg in  $\approx 200 \mu\text{l}$  of PBS), and PBS (200  $\mu\text{l}$ ). DOX HCl and Doxil were purchased from the UCSF hospital pharmacy.

In the experiment intended to determine the antitumor effect of the hydrazone-modified bow tie lacking DOX and the bow-tie dendrimer with DOX attached by means of a stable carbamate linkage, animals were treated on day 8 after tumor implantation with either the hydrazone-linked bow-tie acetone conjugate (238 mg of polymer/kg) or the carbamate-linked bow-tie DOX conjugate (20 mg/kg DOX) in  $\approx 200 \mu\text{l}$  of PBS by tail vein injection. Control mice received 200  $\mu\text{l}$  of PBS.

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