Antivasculature Effects of Doxorubicin-containing Liposomes in an Intracranial Rat Brain Tumor Model

Rong Zhou,1 Richard Mazurchuk, and Robert M. Straubinger2

Department of Pharmaceutical Sciences, University at Buffalo, State University of New York, Amherst, New York 14260-1200 [R. M. S.], and Department of Molecular and Cellular Biophysics Roswell Park Cancer Institute, Buffalo, New York 14263 [R. Z., R. M., R. M. S.]

ABSTRACT

Increased neovascularization and vascular hyperpermeability are integral processes in tumors, and various therapeutic strategies seek to reverse the angiogenic phenotype. Long-circulating liposomes extravasate in tumors such as the rat 9L gliosarcoma and accumulate in perivascular areas. Under such conditions, liposome-encapsulated doxorubicin (DOX) provides ~30% increase in life span, but free DOX is no more beneficial than a saline control. However, the relationship between drug deposition and therapeutic effect is understood poorly. In the present work, magnetic resonance (MR) and functional MR (fMR) imaging were used for noninvasive, serial evaluation of intracranial 9L tumor responses to repetitive doses of free DOX or DOX in sterically stabilized long-circulating liposomes (SSL-DOX). After multiple doses of SSL-DOX, MR imaging revealed the induction of intratumor hemorrhage in 63–75% of rats (n = 8). No hemorrhage was observed by MR imaging after a single dose of SSL-DOX, in normal brain regions in animals treated with free DOX (n = 3) or in saline controls (n = 9). Histological sections from rats sacrificed immediately after MR imaging verified the putative hemorrhagic regions and revealed necrotic and apoptotic tumor cells surrounding the area of the hemorrhage. fMR maps were obtained by comparing paired images acquired during air and Carbogen (7% CO2 and 93% oxygen) breathing. These blood oxygenation level-dependent fMR maps showed enhanced image intensity after both single and multiple doses of SSL-DOX, which suggested increased and progressive vascular permeabilization. The results suggest that the breakdown of tumor vasculature induced by SSL-DOX may arise from the perivascular accumulation of liposomes in tumor and cytotoxic effects on tumor vascular endothelium.

INTRODUCTION

Malignant brain tumors represent a difficult therapeutic challenge. Conventional therapy frequently uses surgery to debulk the tumor mass, followed by radiation and chemotherapy to eliminate residual tumor. However, therapy seldom is curative for many brain tumors. Chemotherapy is hindered by limited drug circulation time, poor penetration of tumors because of inadequate pharmaceutical properties (such as lipid solubility), and poor drug retention, resulting from washout in hyperpermeable areas of tumor or from PGP-mediated drug efflux across the blood-brain barrier. Dose escalation to overcome suboptimal pharmacokinetics and disposition usually is not feasible because of drug toxicity.

Numerous therapies are under development to exploit or inhibit the angiogenic phenotype of solid tumors (1, 2). Both neovascularization and vascular hyperpermeability are integral characteristics for the growth of many tumors and represent potential points of therapeutic intervention. Malignant brain tumors such as Glioblastoma multiforme may be endowed with a hyperpermeable vasculature, as indicated clinically by the observation that regions of the tumor may be accessible to blood-borne contrast agents and radioactive tracers. However, other regions of tumor may be protected by an intact blood-brain barrier.

Liposomes have been shown to extravasate through the compromised vascular bed of many model tumors (3–6), provided the liposome circulation time is adequate for sufficient tumor exposure to the liposomal formulation. Advances in liposome technology such as polymeric coatings (“steric stabilization”) and uniformly small diameter (<0.08 μm) combine to extend liposome circulating lifetime (3, 7). Efficient “remote loading” encapsulation procedures (8, 9) result in high internal concentrations of drug that can remain sequestered within circulating liposomes and, therefore, undergo deposition in tumors as liposomes extravasate. Federal Drug Administration-approved products based on steric stabilization and remote loading exist; one containing DOX3 is known by the name “Doxil” or “Caelyx.”

SSL-DOX enhances the median survival of rats bearing intracranial 9L gliosarcomas tumors by nearly 30% compared with saline controls or animals treated with unencapsulated DOX (P < 0.05; Ref. 10). In parallel, sterically stabilized liposomes labeled with fluorophores undergo deposition in a nonuniform, perivascular pattern in 9L tumors after i.v. injection (10). However, SSL-DOX liposomes show little propensity to interact directly with tumor cells in vitro studies (10), and locally released drug in highly permeable regions of vasculature may exert no greater antitumor effect than the drug given in the unencapsulated form (11). Thus, although enhanced tumor levels of drug can be achieved as the carrier undergoes deposition, the mechanisms of action for drug sequestered in extravasated liposomes is unclear.

The objective of this study was to investigate mechanisms underlying the substantial enhancement in animal life span mediated by treatment with DOX encapsulated in remote-loaded, sterically stabilized liposomes. MR imaging was used for repetitive, noninvasive observation of the therapeutic responses of intracranial tumors in individual rats, so that each animal would serve as its own control. BOLD imaging, a fMR imaging technique, was used for its responsiveness to tumor perfusion and oxygenation level (12, 13). Previously (14), we showed that pharmacological manipulation of blood flow and oxygenation levels resulted in image intensity changes that enabled the discrimination of well-vascularized tumor regions from necrotic regions noninvasively. Here, an imaging protocol involving Carbogen (7% CO2 and 93% oxygen) breathing was implemented to probe the potential vascular effect associated with DOX treatment without adding the potential for confounding drug interactions. In parallel, histological sections of tumors from animals sacrificed immediately after imaging were used to verify and extend the MR imaging findings on the microscopic level.

1 The abbreviations used are: DOX, doxorubicin; BOLD, blood oxygen level-dependent; DSPC, distearoylphosphatidylcholine; PEG-DSPE, polyethylene glycol conjugated to distearoylphosphatidylethanolamine; SSL-DOX, DOX encapsulated in sterically stabilized liposomes; MR, magnetic resonance; T1, longitudinal relaxation time; T2, transverse relaxation time; GRE, gradient echo; SE, spin echo; IMR, functional MR; PGP, P-glycoprotein; Hb, hemoglobin.

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1 Present address: Department of Radiology, University of Pennsylvania, B1 Stellar Chance Labs, 422 Cune Boulevard, Philadelphia, PA 19104-6100.
2 To whom requests for reprints should be addressed, at The Department of Pharmaceutical Sciences, 539 Cooke Hall, University at Buffalo, State University of New York, Amherst, NY 14260-1200. Phone: (716) 645-2844, extension 243; Fax: (716) 645-3693; E-mail: rms@buffalo.edu.
MATERIALS AND METHODS

Materials and methods were described in detail previously (10). Briefly, DOX HCl was obtained as a lyophilized powder (Cetus, Emeryville, CA). Phospholipids (DSPC and PEG-DSPC) were purchased from Avanti Polar Lipids (Alabaster, AL), cholesterol was purchased from Sigma Chemical Co. (St. Louis, MO) and repurified. The rat 9L cell line (designated internally as 9L-72) was obtained from the Brain Tumor Research Center, University of California/San Francisco, and maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum.

Preparation of Long-circulating DOX-containing Liposomes. Small, sterically stabilized liposomes containing DOX (SSL-DOX) were prepared from DSPC:cholesterol:PEG-DSPC in a 9:5:1 molar ratio. Drug was incorporated into preformed liposomes by minor modifications of a remote loading procedure (9) detailed previously (10). Briefly, the lipids (in solvent) were dried to form a thin film and were hydrated with 1 ml of ammonium sulfate (250 mM [pH 5.5]) per 10 μmol lipid. The liposomes were extruded (≥3 times through 0.08 μm polycarbonate filters at 60°C) and the excess ammonium sulfate was removed by dialysis against isotonic sucrose. DOX dissolved in isotonic sucrose (10% w/v) at 2 mg/ml was incubated with the preformed liposomes for 1 h at 65°C, at a drug:lipid ratio of ~1:5 (mol:mol). Generally, >90% of the initial DOX was encapsulated. Liposomes were dialyzed against isotonic sucrose to remove residual unencapsulated drug. The phospholipid concentration was determined by phosphorous assay (15), and the DOX concentration was determined by spectrophotometry after solubilization of liposomes in acidic methanol.

Tumor Implantation. Intracranial 9L tumors were established by stereotaxic injection of ~4 × 10^6 9L cells (in ≥5 μl) into the caudate/putamen region of anesthetized male Fisher 344 rats (Harlan Sprague Dawley, Indianapolis, IN) weighing 130–160 grams. The procedure was described previously (10) in detail. The injection location was 1.5 mm anterior- and 2.4 mm lateral to Bregma, and 4.5 mm beneath the exposed dura. The burr hole in the skull was plugged with Gelfoam (Pharmacia and Upjohn, Kalamazoo, MI), and the scalp was sutured if animals were to be used for MR imaging. Protocols involving animals conformed to the guidelines of the Institutional Animal Care and Use Committees (IACUC) of the State University New York at Buffalo and the Roswell Park Cancer Institute, and were approved in advance.

Therapy of 9L Tumors with DOX-containing Liposomes. Rats bearing 9L brain tumors were treated via tail vein injection with DOX dissolved in saline (“free DOX”) or encapsulated in sterically stabilized liposomes (SSL-DOX). A control group received saline only. Treatment began 7 days after tumor implantation, and was given as three weekly i.v. injections of 5.67 mg/kg DOX. The cumulative dose of DOX (17 mg/kg) represented the tumor implantation, and was given as three weekly i.v. injections of 5.67 mg/kg DOX encapsulated in small, sterically stabilized liposomes (SSL-DOX) resulted in a nearly 30% life-span extension in rats bearing intracranial 9L tumors (10). No dose of free DOX, including or exceeding the maximum tolerated dose, extended life span relative to saline-treated controls. Treatment was initiated 7 days after implantation of 9L cells, a time at which well-vascularized tumors can be observed histologically in the brain (data not shown).

Serial MR imaging was undertaken to enable intracranial tumor volume measurements, so that dose and scheduling of treatment could be optimized. By MR imaging, 9L tumors become observable as a distinct entity 9–11 days after implantation (data not shown). Therefore, imaging was initiated on day 9 and was repeated every 2–3 days throughout the 3-week treatment cycle.

Representative T1-weighted GRE images of 16-day intracranial tumors are shown in Fig. 1. The tumor was visualized as a hypointense entity (darker than the surrounding tissues), and anatomical details of the brain were well visualized (Fig. 1A). For each of the three treatment groups, a few animals were imaged serially in a preliminary experiment, to evaluate the feasibility of repetitive tumor volume measurements during treatment.

For saline-treated controls, tumors progressed to a volume of ~5 mm^3 by day 21 after inoculation. Thereafter, tumor volume increased...
more rapidly; those animals that survived were symptomatic and bore 18–20-mm³ tumors by day 25 (data not shown). For animals treated weekly with free DOX, tumor progression roughly paralleled that of control animals, although drug toxicity was apparent by day 15–20 (data not shown), just after the second DOX dose (given on day 14). For animals treated with SSL-DOX, tumor volume initially paralleled that of saline-treated controls but appeared to progress more rapidly after the second dose of SSL-DOX (given on day 14). The apparent tumor volume was nearly 16 mm³ on day 21, ~3-fold greater than that of saline controls at the equivalent time point (data not shown).

Fig. 1B shows a slice from a representative T1-weighted GRE image series from an animal treated with SSL-DOX. The anatomical location is similar to that shown in Fig. 1A. The image in Fig. 1B was acquired 48 h after the second (day 14) injection of 5.67 mg/kg liposomal DOX. A severe hypointense artifact was observed in the image, and this artifact confounded tumor volume measurements. Such artifacts typically arise from vascular breakdown and the degradation products associated with hemorrhage, including deoxyHb, metHb, and hemosiderin. These compounds are paramagnetic, and sharp susceptibility gradients or local magnetic field inhomogeneities would be generated by the interface of these paramagnetic substances with the more diamagnetic surrounding tissues. Hemorrhage was verified by histological examination of tissues taken immediately after image acquisition (data not shown).

Because of the sensitivity of GRE images to local magnetic field inhomogeneities and the distortions observed in the tumor region (Fig. 1B), SE (SE) imaging sequences were used for more detailed investigation of the effects of DOX treatment on tumor vascular permeability. It was expected that the 180° refocusing pulse of the SE imaging sequence, which makes SE images more immune to local field inhomogeneity, would reduce image distortion induced by hemorrhage.

Fig. 2 shows T2-weighted SE images of representative control (Fig. 2A and B)- and SSL-DOX (Fig. 2C and D)-treated animals. Because of the different mechanism by which contrast is generated in SE images, the tumor appears as a hyperintense entity with a distinct boundary (see Fig. 2A). No evidence of hemorrhage appears in Fig. 2A or 2C (images were acquired 24 h before the second weekly treatment with saline or SSL-DOX, respectively). Forty-eight h after administration of saline, no hemorrhage was observed in tumors of control animals (Fig. 2B) or animals receiving free DOX (data not shown). In contrast, for animals treated with a second dose of 5.67 mg/kg SSL-DOX (on day 14), marked changes were observed 48 h after treatment. Fig. 2D shows an image slice transecting the tumor of the same animal as in Fig. 2C, but acquired on day 16. A hypointense region appears within the tumor and suggests the induction of hemorrhage. Analysis of brain histological sections, obtained immediately after imaging, confirmed hemorrhage in an area corresponding to the hypointense region on the MR image (Fig. 3, D and E). Compared with the GRE images (Fig. 1), signal loss attributable to hemorrhage (i.e., in the hypointense area, Fig. 2D) appeared both more confined in T2-weighted SE images and more conspicuous against the hyperintense background of the tumor tissue.

A systematic study was undertaken to investigate the occurrence of hemorrhage as a function of time, treatment regimen, and dosage form. Individual animals were evaluated serially and repetitively by MR imaging, and scored for the appearance of hemorrhage. Histological verification was performed on a subset of animals, or after death in those animals that became moribund.

Table 1 compares the occurrence of hemorrhage in saline-treated controls, and in animals treated with free- or liposome-encapsulated DOX. Sixty-three % (5 of 8) of rats treated with SSL-DOX showed hemorrhage only after the second weekly treatment; in one additional animal, hemorrhage appeared after the third weekly injection of SSL-DOX. Animals bearing hemorrhage appeared asymptomatic and, as a group, showed a significant extension of life span (Table 1), consistent with previous observations (10). Hemorrhage was not observed in the contralateral (tumor-free) hemisphere or after a single treatment with SSL-DOX (data not shown).

No hemorrhage was observed in saline- or free DOX-treated animals (Table 1); these animals became symptomatic from tumor progression, and had an ~30% shorter life span than animals treated with SSL-DOX, consistent with previous results (10). Some animals in the free DOX group were lost to MR evaluation before the day-16 evaluation, because of a combination of drug toxicity and tumor progression.

For those animals treated with SSL-DOX that did not show hemorrhage (25–37%), tumor volumes appeared in MR images to be larger than those of other treatment groups (data not shown). This finding was paradoxical, considering the extended life span of animals treated with SSL-DOX. However, with images acquired using the same scanner settings and displayed under the same dynamic range, images of SSL-DOX-treated tumors (Fig. 2C, 6 days after the first weekly treatment), appeared brighter than those of the controls (Fig. 2A, corresponding saline control animal). This finding indirectly suggests an increase in T2 relaxation time in the tumor and is consistent with the hypothesis that these tumors may have higher water...
content as a result of increased vascular permeability. Therefore, although hemorrhage was observed exclusively after the second or third treatment of SSL-DOX, a difference in T2 relaxation times after the first treatment would suggest a progressive and cumulative increase in tumor vascular permeability with SSL-DOX treatment, leading to ultimate vascular breakdown and hemorrhage.

To probe the possible vascular effects associated with SSL-DOX treatment, a BOLD fMR imaging protocol (14) was modified and implemented. T2-weighted SE images were acquired from the same anatomical location of a single rat acquired with animal breathing (A) room air and (B) Carbogen (7% CO2 and 93% O2; Fig. 3B). Fig. 3C shows a functional map of the tumor region computed from images in Fig. 3A and B, as described in “Materials and Methods,” and overlaid on the T2-weighted image. The pseudocolor scale in Fig. 3C represents the percent signal intensity increase observed after exposure of the animal to Carbogen. The hypointense region within the tumor, verified histologically as hemorrhagic (Fig. 3D), shows a large (>20%) intensity increase in the fMR map (Fig. 3C). Because the microvasculature density in this region (1.4 ± 0.5/mm²) is much lower than in the tumor periphery (3.8 ± 1.8/mm²; Ref. 14), the greatest contribution to increased intensity on the fMR map is the increased water diffusion through a highly permeable vasculature (in response to Carbogen-induced increased cerebral blood flow), rather than the BOLD effect.

This finding suggests that the fMR map displays increased water flux through a highly permeable tumor vasculature in the hemorrhagic region, a response to Carbogen-induced increases in cerebral blood flow.

Foci of necrotic cells and vasogenic edema were observed around the hemorrhagic region (Fig. 3E), along with large numbers of neutrophils and lysed RBCs in the central hemorrhage area. The hypointense region visualized on Fig. 3 appeared to occupy a larger portion of the tumor than actually observed as hemorrhagic in histological sections (Fig. 3D). This effect arises primarily from the paramagnetic nature of hemorrhage degradation products accumulated in the region, which induce field inhomogeneity and a resulting loss in signal in these areas.

A large intensity increase also was observed in the fMR map in the area peripheral to the tumor (Fig. 3C), which was observed histologically as a well-vascularized and invasive tumor region (Fig. 3D). This fMR intensity change most likely results from increased oxygenation in capillaries and small veins (BOLD effect) during Carbogen breathing (14).

**DISCUSSION**

The 9L rat brain tumor model possesses several characteristics of lethal tumors such as Glioblastoma multiforme (17). In this model, a small number of cells injected stereotaxically form an invasive, well-vascularized intracranial tumor that has minimal necrosis throughout most of the animals’ life spans. In addition, 9L cells express PGP (18), which renders them resistant to a wide range of anticancer agents, including DOX. The PGP status of 9L cells is consistent with our previous observations (10) and present observation that free DOX exerts essentially no antitumor effect.
Previous studies in various tumor models have shown that sterically stabilized liposomes similar to those used here mediate both large increases in DOX deposition and substantial increases in survival (3, 19, 20); in a malignant fibrous histiocytoma brain tumor model (21), SSL-DOX provided a 14-fold higher DOX concentration in tumor (compared with free DOX), as well as with sustained intratumor drug concentrations. An 89% increase in survival (relative to untreated controls) was observed in rats treated with SSL-DOX, compared with a 23% increase in survival with free DOX (21). The more modest therapeutic effect of SSL-DOX observed with 9L tumors (Table 1; Ref. 10) and the lack of effect of free DOX are consistent with the drug-resistant and invasive phenotype of the 9L model.

MR imaging provides an opportunity to observe tumor responses to therapy in a serial fashion, using each animal as its own control. Here, MR imaging revealed a previously unreported response to SSL-DOX: disseminated hemorrhage within brain tumors of 63–75% of rats receiving multiple treatments of SSL-DOX. Hemorrhage was not observed in the normal tissue of the contralateral hemisphere, in animals treated with free DOX, nor in those receiving just a single dose of SSL-DOX. In our experience, spontaneous hemorrhage in 9L tumors is seldom observed, particularly in the less advanced tumors imaged here; analysis of the control group (Table 1) verifies that hemorrhage was absent in saline-treated animals.

Drug effects on tumor cells or the tumor vasculature may underlie the hemorrhage observed after treatment with DOX-containing liposomes. Our working hypothesis is that the production of hemorrhage arises from a drug delivery mechanism that exploits increased tumor vascular permeability and neovascularization as well as specific characteristics of the drug-carrier system. The observation that most of the hemorrhaging occurred in the central region of the tumor is consistent with the up-regulation of VEGF (vascular endothelial growth factor) and other modulators of vascular integrity expressed in hypoxic regions of the tumor (22). The vasculature in these areas tends to be fenestrated and permeable to liposomes of the size range used here (3–6, 23). It should be vulnerable to attack after the local deposition of cytotoxic drugs such as DOX.

The perivascular accumulation of SSL-DOX, observed in intracranial 9L and other tumors (5, 10, 24), may mediate a direct effect of DOX on the tumor vasculature: after liposome extravasation and accumulation in regions of neovascularization or vascular repair, DOX released from liposomes may inhibit proliferating endothelial cells. Alternatively, DOX may induce vasculature damage by generating reactive oxygen species; the quinone moiety of DOX is a substrate of the endothelial isoform of nitric oxide synthase (eNOS), and it can be converted to semiquinone radical, resulting in decreased production of nitric oxide and increased formation of superoxide anions, peroxynitrite, and hydrogen peroxides (25).

An alternative mechanism of hemorrhage is that the vascular effect is secondary to tumor cell death. The secretion of various growth factors such as VEGF, which support the endothelial cells and/or pericytes of the vessels (26, 27), may diminish as tumor-deposited drug kills tumor cells, resulting in vessel collapse. However, although apoptotic cells and the infiltration of inflammatory cells were observed, no histological evidence was found of massive cell death surrounding hemorrhagic regions.

The onset of hemorrhage, which appeared only after multiple doses of SSL-DOX, may also result from special properties of the drug-carrier combination. Our initial effort at tumor volume measurements were confounded by treatment-related changes in the tumor, and the most rapid apparent volume increase was observed in the longest-surviving rats (Ref. 10; Table 1). However, the changes observed in MR as volume increases are consistent with effects associated with increased tumor vascular permeability caused by treatment with SSL-DOX. The data suggest an increase in interstitial water content in the peripheral, invasive regions of the tumor that were not well demarcated by conventional 1H MR imaging protocols. Interestingly, these changes in permeability were induced by SSL-DOX, but not by free DOX. Thus, although hemorrhage was not observed after the first dose of liposomal drug, the initial SSL-DOX treatment may exert a permeabilizing effect on the vasculature, perhaps enabling greater extravasation and vascular damage by subsequent doses of liposomes. Such a mechanism would be consistent with our previous observation of nonuniform perivascular deposition of liposomes in 9L brain tumors (10).

fMR techniques offer the possibility of observing biochemical and physiological responses of tumors in unprecedented detail (12, 13). Previously we showed that a “vascular challenge,” accomplished by altering hemodynamics with acetazolamide, provided a basis for fMR discrimination of the invasive, vascularized regions of 9L tumors from normal brain tissue (14). Here, a protocol involving Carbogen breathing was implemented to simplify the imaging protocol and eliminate possible pharmacological interactions. The MR signal in T2-weighted SE images increased within regions of the tumor in response to Carbogen breathing. These changes resulted from the BOLD effect and/or perturbations of flow. Hb transitions between oxy- and deoxy-Hb state occur throughout the circulatory tree, with higher concentrations of deoxyHb in venous blood (~30%) than in arterial blood (~5%). The susceptibility difference between oxyHb, which is diamagnetic, and deoxyHb, which is less diamagnetic, provides the physical basis of BOLD fMR techniques (28, 29). DeoxyHb, when present at high concentration, would induce local susceptibility effects that result in signal loss on MR images. Breathing of CO2 would increase cerebral blood flow (30), elevate the oxygenation of blood in capillaries and veins, and change the ratio of oxyHb:deoxyHb in these compartments. Consequently, regions associated with capillaries and veins should have higher image intensities during CO2 breathing than during room air breathing. Indeed, the tumor periphery was visualized on fMR maps as an area of high-intensity change (~20%), consistent with our previous finding (14) that the signal intensity increase results from increased oxyHb in the well-vascularized tumor periphery. Interestingly, the hypo-intense regions on T2 images (identified histologically as hemorrhagic) were visualized on fMR maps as regions of low intensity increase (~20% from baseline). Increased vascular permeability may also contribute significantly to the intensity increase: as blood flow increases, more water molecules may diffuse into the hemorrhagic region. Thus, the fMR imaging approach used here, provides functional information such as perfusion and vascular permeability for a region that otherwise would not be well differentiated from necrosis on conventional MR images. New approaches such as dynamic contrast-enhanced MR imaging, using Gd-DTPA (gadolinium-diethyleneetriamine-pentaacetic acid) as a diffusible extracellular tracer, also are emerging as promising tools for monitoring permeability and/or perfusion (31, 32). Dynamic MR imaging recently has been applied to the functional analysis of 9L tumor neovascularization and has been validated using immunohistological analysis of vascular architecture at the microscopic level (33).

In conclusion, the ability of liposomes to extravasate through the leaky vasculature in tumor, coupled with poor lymphatic drainage, results in perivascular accumulation of liposomes. Although they may act as drug reservoirs and provide sustained, local release of drug, the vascular effect induced by SSL-DOX appears to involve additional mechanisms. The unique biodistributional properties of the liposome carrier may combine with the altered behavior of DOX encapsulated by remote loading, to confer a new, antivascular mechanism of action on the drug-carrier complex. The applicability of this mechanism to other tumor types, its exploitability for enhancing the subsequent
delivery of other chemotherapeutic agents, and the implications for alternative dosing regimens of this clinically approved formulation warrant further investigation.

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