Magnetic Resonance Imaging of Temperature-Sensitive Liposome Release: Drug Dose Painting and Antitumor Effects

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Background

In preclinical studies, lysolipid-based temperature-sensitive liposomes (LTSLs) containing chemotherapy drugs administered in combination with local hyperthermia have been found to increase tumor drug concentrations and improve antitumor efficacy of the drugs. We used a novel magnetic resonance imaging (MRI) method to measure the temporal and spatial patterns of drug delivery in a rat fibrosarcoma model during treatment with LTSLs containing doxorubicin and an MRI contrast agent (manganese) (Dox/Mn-LTSLs) administered at different times with respect to hyperthermia.

Methods

Rats bearing 10- to 12-mm fibrosarcomas (n = 6–7 per group) were treated with Dox/Mn-LTSLs (at a dose of 5 mg doxorubicin/kg body weight) before and/or during 60 minutes of local tumor hyperthermia administered via a catheter inserted at the center of the tumor. Drug distribution was monitored continuously via MRI. Magnetic resonance changes were used to calculate intratumoral doxorubicin concentrations throughout treatment. Tumors were monitored until they reached five times their volume on the day of treatment or 60 days. Doxorubicin concentrations and times for tumors to reach five times their volume on the day of treatment were analyzed using the Kruskal–Wallis test and the Kaplan–Meier product-limit method, respectively. All statistical tests were two-sided.

Results

Administration of Dox/Mn-LTSLs before, during, and both before and during hyperthermia yielded central, peripheral, and uniform drug distributions, respectively. Doxorubicin accumulated more quickly and reached higher concentrations in the tumor when Dox/Mn-LTSLs were administered during hyperthermia than when administered before hyperthermia (rate: 9.8 versus 1.8 µg/min, difference = 8.0 µg/min, 95% confidence interval [CI] = 6.8 to 12.8 µg/min, P = .003; concentration: 15.1 versus 8.0 ng/mg, difference = 7.1 ng/mg, 95% CI = 3.6 to 10.6 ng/mg, P = .028). LTSL administered during hyperthermia also yielded the greatest antitumor effect, with a median time for tumors to reach five times their volume on the day of treatment of 34 days (95% CI = 30 days to ∞) compared with 18.5 days (95% CI = 16 to 23 days) for LTSL before hyperthermia and 22.5 days (95% CI = 15 to 25 days) for LTSL before and during hyperthermia.

Conclusions

In this rat fibrosarcoma model, LTSLs were most effective when delivered during hyperthermia, which resulted in a peripheral drug distribution.

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The efficacy of most systemic chemotherapeutics is limited by tissue toxicity and by physiologic barriers that prevent the delivery of drug to the tumor. Polymer-based and liposomal drug delivery systems have been designed to increase tumor drug levels while limiting systemic drug exposure (1). For example, in human xenograft-bearing mice treated with doxorubicin-containing lysolipid-based temperature-sensitive liposomes (Dox-LTSLs; Fig. 1), tumor doxorubicin concentrations were up to 30 times higher than those of mice treated with free drug, resulting in dramatic improvements in antitumor efficacy (2,3). Dox-LTSLs, which are now in phase I clinical trials (26), achieve complete release of their contents within 20 seconds when exposed to mild local hyperthermia (40–42 °C) (4). In vitro studies suggest that this release occurs through membrane pores that form during the melting phase transition of the lipid bilayer (5) (Fig. 1).

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**CONTEXT AND CAVEATS**

**Prior knowledge**
Temperature-sensitive liposomes rapidly release their contents when heated to 41.3 °C and thus have the potential to target delivery of systemic chemotherapy to tumors when combined with local hyperthermia. However, the optimal timing of local hyperthermia with respect to temperature-sensitive liposome administration is not known.

**Study design**
In vivo therapeutic study in a rat fibrosarcoma model.

**Contribution**
A novel MRI method was used to show that drug-containing temperature-sensitive liposomes were most effective when intravenously injected while the tumor was being heated via a centrally placed heating catheter.

**Implications**
The timing of local hyperthermia with respect to the administration of drug-containing temperature-sensitive liposomes can be used to control intratumoral drug distribution.

**Limitations**
The heating method used in this study is not applicable to humans. Rat local tumor drug kinetics may differ from human local tumor drug kinetics.

The improved efficacy of Dox-LTSLs relative to free doxorubicin and non–temperature-sensitive Dox liposomes reflects their unique mechanism of action. In human xenografts implanted in mouse dorsal skinfold chambers, the antitumor effect of Dox-LTSLs was associated with decreases in tumor blood flow and microvessel density, which were not observed with free doxorubicin (6). This antivascular action was most likely due to the predominantly extravascular release of doxorubicin from LTSLs that were delivered during local hyperthermia, which resulted in the exposure of the tumor vascular and/or perivascular cells to very high drug concentrations. The rapid release rate of LTSLs theoretically permits local (i.e., tumor) targeting of intravascular release. This paradigm is markedly different from that of other drug carriers, which have been designed to extravasate through the permeable tumor vasculature and release their contents slowly in the interstitium (1,7).

A novel magnetic resonance imaging (MRI) technique has been used to observe in vivo content release from Dox-LTSLs that contain manganese (Mn) as a contrast agent (Dox/Mn-LTSLs) (8). Local tissue doxorubicin concentrations were estimated from the shortening of MR T1 relaxation times. In a rat fibrosarcoma model, T1-based tumor doxorubicin concentrations were correlated with doxorubicin concentrations measured in tumor samples by high-performance liquid chromatography (HPLC) and histologic fluorescence (9). In these studies (8,9), Dox/Mn-LTSLs administered during local hyperthermia released their contents immediately as they entered the heated tumor from the peripheral blood vessels. This finding suggested that the pattern of drug delivery with LTSLs may depend on the tumor perfusion pattern and the temperature profile at the time of LTSL injection. The use of different hyperthermia protocols could permit intratumoral drug distribution to be controlled in real time, a concept we call “drug dose painting.”

In this study, we used MRI to measure temporal and spatial patterns of drug delivery in a rat fibrosarcoma model during treatment with Dox/Mn-LTSL and hyperthermia administered with different schedules. Our goal was to investigate the relationships among temperature profile, tumor drug delivery pattern, and antitumor effect.

**Materials and Methods**

**Materials**
The following phospholipids were purchased from Avanti Polar Lipids Inc (Alabaster, AL): 1,2 dipalmitoyl-sn-glycerol-3-phosphocholine (DPPC); 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine (MSPC); and 1,2 distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000). Sephadex G-50 medium was purchased from GE Healthcare (Piscataway, NJ). HEPES sodium salt, manganese sulfate monohydrate, sucrose, ammonium phosphate dibasic, perchloric acid, and ammonium molybdate were purchased from Sigma-Aldrich (St Louis, MO). Doxorubicin was purchased from Pharmacia and Upjohn Company (Kalamazoo, MI).

**Liposome Preparation**
Temperature-sensitive liposomes were prepared from DPPC, MSPC, and DSPE-PEG2000, which were combined in a molar ratio of 90:10:4 as previously described (8,10). The phospholipids were combined in chloroform, and the solvent was removed using nitrogen gas and high vacuum. The dried samples were hydrated with 300 mM manganese sulfate (MnSO4) at pH 3.5 to a lipid concentration of 80 mg/mL. Hydration was performed at 55 °C for 30 minutes. The hydrated lipid vesicles were extruded 10 times through stacked polycarbonate filters of 0.1- and 0.08-μm pore size at 55 °C by using a water-jacketed extruder (Northern Lipids Inc, Vancouver, Canada). The phospholipid concentration was
measured using the Fiske and Subbarow phosphate assay, as previously described (11,12). Briefly, 70% perchloric acid was added to liposome samples as well as to phosphate dibasic standard solutions (concentration range = 0.25–1 mM). The mixtures were heated at 180–200 °C for 2 hours. After cooling, Fiske reagent and ammonium molybdate were added, and the samples were reheated to 100 °C for 20 minutes. Absorbance at 820 nm was linearly related to phosphate concentration and was measured for all samples and standards.

Production of Liposomes Containing Doxorubicin and Manganese

Liposomes (1-mL aliquots) were fractionated on 20-mL Sephadex G-50 columns that had been pre-equilibrated with 300 mM Sucrose/20 mM HEPES buffer (pH 7.5) to remove unencapsulated MnSO 4 . Doxorubicin was then added to the fractionated liposome solution at a drug-to-lipid ratio of 0.05:1 (wt : wt), and loading of doxorubicin into liposomes was conducted at 37 °C for 80 minutes (8,9,10). Liposomes were fractionated again on Sephadex G-50 columns that had been pre-equilibrated with HEPES buffer to remove unencapsulated doxorubicin, and the concentration of the liposome-encapsulated doxorubicin was estimated using fluorimetry with excitation and emission wavelengths of 480 and 550 nm, respectively.

In Vivo Therapeutic Studies in Rats

Tumor model. The tumor model used in this study was a fibrosarcoma (FSA-1, provided by Bull J, The University of Texas M. D. Anderson Cancer Center, Houston, TX) that was originally isolated from the subcutis of rats that were given the carcinogen methylcholanthrene and was maintained by serial transplantation (8,9,13). The fibrosarcoma was transplanted subcutaneously in the flanks of 8-week-old female Fischer 344 rats (Charles River Laboratories, Wilmington, MA) under anesthesia using intraperitoneal ketamine (9 mg/kg body weight) and xylazine (10 mg/kg body weight). Tumors were allowed to grow for approximately 2 weeks to a diameter of 10–12 mm before treatment.

Treatment protocols. Tumor-bearing rats were anesthetized with an intraperitoneal injection of pentobarbital (45 mg/kg) and randomly assigned to one of the following eight treatment groups (n = 9–10 rats per group): saline only (control), hyperthermia alone, free doxorubicin alone, free doxorubicin during hyperthermia, Dox/Mn-LTSL alone, and Dox/Mn-LTSL with hyperthermia given according to one of three protocols. In protocol 1, hyperthermia was initiated 15 minutes before Dox/Mn-LTSL was administered so that a thermal steady state was reached before the drug was delivered (Dox/Mn-LTSL during hyperthermia). In protocol 2, Dox/Mn-LTSL was administered 15 minutes before the initiation of hyperthermia (Dox/Mn-LTSL before hyperthermia). In protocol 3, half of the dose of Dox/Mn-LTSL was administered 15 minutes before initiation of hyperthermia and the other half was administered after thermal steady state was reached (i.e., 15 minutes after the initiation of hyperthermia; Dox/Mn-LTSL split dose).

Each group of rats (except the control group) received an equivalent dose of 5 mg doxorubicin/kg body weight by intravenous injection and/or 1 hour of hyperthermia. Local hyperthermia was delivered by means of an 18-gauge catheter that was placed through the center of the tumor and through which heated water (~50 °C, 1.8 mL/s) was passed. This magnetic resonance-compatible heating system provides a reproducible radial temperature distribution that reaches thermal steady state within 15 minutes (8). The average temperatures were previously determined to be 45–46 °C adjacent to the catheter and 38.5–39.5 °C at the tumor border (8). A rectal thermistor was used to monitor the body temperature of the rats during treatment and during MRI (14).

For each treatment group, three rats were used to measure doxorubicin concentrations in tumors and hearts by HPLC. The rats were killed by carbon dioxide asphyxiation 90 minutes after Dox/Mn-LTSL injection (an interval similar to that between injection and the time the final magnetic resonance images were obtained), and their tumors and hearts were immediately harvested, snap frozen, and stored at −80 °C. The remaining 6–7 rats in each treatment group were included in the survival analysis. Magnetic resonance images were acquired before, during, and after therapy for rats in the three Dox/Mn-LTSL plus hyperthermia groups. Final images were taken after tumors cooled to room temperature. Treatment groups that did not receive Dox/Mn-LTSL were not imaged. After therapy, the rats were monitored as they recovered from anesthesia. Tumor volumes were measured three times per week for 60 days or until the tumor volume reached five times the volume on the day of treatment. At these endpoints, the rats were killed by carbon dioxide asphyxiation. The 60-day cut point was chosen for comparison to previous studies with Dox/LTSL (2,3). The five times the initial volume cut point was chosen based on guidelines from the Institutional Animal Care and Use Committee. No rats were killed before the specified endpoints were reached. This animal protocol was approved by the Duke University Animal Care and Use Committee.

Magnetic resonance imaging protocol. MRI data were acquired before and during therapy with the use of a 2T 30-cm-diameter bore magnet (Oxford Instruments, Oxford, U.K.) and a Sigma magnetic resonance console (GE Healthcare, Waukesha, WI). A series of axial T 1 -weighted images were acquired using a three-dimensional spoiled gradient-echoed echo acquisition at flip angles of 2°, 5°, 12°, 17°, 20°, 33°, 45°, and 70° to estimate the initial T 1 map (8,9). These images were acquired by using the following parameters to produce the greatest T 1 contrast: repetition time = 22 ms; echo time = minimum; matrix = 256 × 256 × 28; field of view = 6 cm; slice thickness = 1 mm; bandwidth = 15.63 Hz/pixel; and number of excitations = 1. Images acquired using these parameters had a voxel size of 234 × 234 × 1000 μm. Magnetic resonance scans during and after Dox/Mn-LTSL treatment were performed with a flip angle of 33° and a scan time of 90 seconds. These axial scans were acquired continuously throughout therapy by starting a new scan every 90 seconds (8,9).

Analysis of Magnetic Resonance Images to Estimate Doxorubicin Concentration

Magnetic resonance images were analyzed to estimate the doxorubicin concentration for each voxel at each time point, as previously described (8,9). Briefly, the initial T 1 and S 0 values for each voxel...
were obtained by fitting the signal intensity at variable flip angles (α) to the following equation:

$$\text{signal} = S_0 \left( 1 - \cos(\alpha) \exp\left( -\frac{T_E}{T_1} \right) \right)$$

where $S_0$ accounts for the proton density and $T_1$ effects (15). Each subsequent image was converted to a $T_1$ map with the use of the new signal values and the initial values of $S_0$. Signal enhancement was assumed to be caused by $T_1$ shortening (15). We used the linear relationship between $1/T_1$ and the MnSO$_4$ concentration described in the equation

$$\frac{1}{T_1} = R_1 \left( C_{\text{Mn}^{2+}}^\text{dynamic} - C_{\text{Mn}^{2+}}^\text{0} \right),$$

to convert changes in $T_1$ to manganese concentrations. The relaxation rate of manganese ($R_1$, in mM$^{-1}$s$^{-1}$) is dependent on the temperature and encapsulation state of the manganese ions (15). Therefore, to calculate $R_1$ values for each voxel, we used previously published (8) in vivo temperature measurements for this tumor model, and we assumed complete release of manganese from the liposomes. Manganese concentrations (mM) were converted to doxorubicin concentrations ($\mu$g/mL) by multiplying $C_{\text{Mn}^{2+}}^\text{dynamic}$ by the ratio of encapsulated doxorubicin to encapsulated manganese in the injected liposomes. The final $T_1$-based maps of doxorubicin concentrations were compiled as images similar to the magnetic resonance images, with the concentration represented by the intensity at each voxel (8,10). This conversion assumes that doxorubicin and manganese remain colocalized after they are released from the liposome.

Spatial and Temporal Analysis of Drug Delivery

We used the final $T_1$-based maps of doxorubicin concentrations in the tumors to perform a quantitative analysis of the spatial distribution of doxorubicin in the tumor. For each rat, eight radial line profiles of doxorubicin concentrations were acquired from each of the 10 central tumor slices along lines that radiated, at 45° angles, from the central catheter to the edge of the tumor (as illustrated in Fig. 2, A). The lengths of these lines were normalized, such that the catheter had a radial value of zero and the edge of the tumor had a radial value of 1.0. We calculated the mean doxorubicin concentration at each radial distance for each tumor and then for each treatment group. For each tumor, the area under the curve (AUC) in the peripheral portion of the tumor (defined as radial values from 0.5 to 1.0) was compared with the AUC in the central portion of the tumor (defined as radial values from 0 to 0.5) to calculate the doxorubicin distribution (i.e., AUC) ratio. The AUC ratio is thus greater than 1 if the doxorubicin is concentrated at the edge of the tumor and less than 1 if the doxorubicin is concentrated at the center of the tumor.

For each rat, we calculated the total amount of doxorubicin that was delivered to the whole tumor at each time point by multiplying the volume of each voxel (0.055 mm$^3$) by the concentration of doxorubicin and then summing the doxorubicin levels over all voxels. Error bars indicate 95% confidence intervals (CIs) among animals in a given group at a given time point. The initial rate of doxorubicin accumulation in the tumor (micrograms of doxorubicin per minute) was averaged over the first 3 minutes of combined treatment for each rat and each treatment group.

High-Performance Liquid Chromatography Analysis of Tumor Doxorubicin Concentration

Frozen tumors and hearts were thawed and homogenized, and doxorubicin concentrations were measured by HPLC as previously described (2,16). In brief, doxorubicin was extracted from the homogenates using chloroform and silver nitrate. The organic phase was collected, dried, and reconstituted in isopropanol. Doxorubicin concentration was measured by fluorimetric emission at 550 nm following HPLC separation. A standard concentration set was prepared from homogenates of tumors from the control group that were spiked with known amounts of doxorubicin.

Statistical Analysis

Descriptive summary statistics are expressed as mean values with 95% confidence intervals, except for the time to five times tumor volume data, which are expressed as median values with 95% confidence intervals. To avoid assuming a normal distribution, nonparametric or semiparametric statistical methods were used. Group comparisons were conducted with the Kruskal–Wallis test for more than two groups and the Wilcoxon test for between-group differences. For the antitumor efficacy data, the primary endpoint (time to reach five times the original tumor volume) was analyzed by the Kaplan–Meier product-limit method, using the Wilcoxon rank test for between-group differences. Censoring was taken into account for animals that showed complete regression and were therefore killed at 60 days. The Cox proportional hazards regression technique for growth time was also employed to explore whether $T_1$-based doxorubicin concentration was statistically significantly associated with the time to five times the initial tumor volume. This regression model assumed a different baseline hazards function for each treatment group, thereby avoiding the assumption of proportional hazards across different treatment groups. For all tests, $P$ values less than .05 were considered statistically significant. Because these analyses were exploratory in nature, no adjustments were made for multiple comparisons. SAS (version 9.1; SAS Institute Inc, Cary, NC) was used to implement all the statistical analyses reported here. All statistical tests were two-sided.

Results

Magnetic Resonance Images

Figure 2 shows examples of final axial magnetic resonance images resulting from each Dox/Mn-LTSVs plus hyperthermia protocol, with the fibrosarcoma positioned in the upper left corner. The slices were chosen to show each tumor at its greatest diameter; the heating catheter was located near the center of each tumor. The enhanced (i.e., white) areas within the tumors represent free manganese (i.e., manganese released from the liposomes). Full sets of magnetic resonance images over the 75-minute treatment period for these three examples were compiled as Supplementary Movies 1–3 (available online). Rats injected with Dox/Mn-LTSVs during steady-state
hyperthermia displayed a peripheral enhancement pattern near the edge of the tumor, similar to what had been observed previously (8) (Fig. 2, A). Supplementary Movie 1 (available online) shows that the liposomes released their contents quickly into the periphery of the tumor within 10 minutes of injection. Rats injected with Dox/Mn-LTSLs before hyperthermia displayed signal enhancement (i.e., content release; white) at the edge of the tumor (A); LTSLs administered before hyperthermia resulted in central enhancement (B); and LTSLs administered in two equal doses, half before hyperthermia and the remainder after steady-state hyperthermia was reached, resulted in uniform enhancement (C). (D) $T_1$-based mean tumor doxorubicin concentration (nanograms per milligram of tissue) after treatment with LTSLs during hyperthermia, shown as a function of the normalized tumor radius for each rat. The bold profile is for the tumor shown in (A). Mean values are for 80 line profiles from each rat. (E) Mean doxorubicin concentration (nanograms per milligram of tissue) profiles for each of the three therapeutic groups as a function of the normalized tumor radius ($n = 6–7$ rats per group). Vertical lines in (D) and (E) correspond to 95% confidence intervals.

**Intratumoral Distribution of Doxorubicin**

We next used $T_1$-based maps of doxorubicin concentrations (data not shown) to examine the intratumoral distribution of doxorubicin in rats that had been treated with each of the three Dox/Mn-LTSL plus hyperthermia protocols. The areas of high doxorubicin concentration were similar to the enhanced areas in the magnetic resonance images. Each of the six rats that received Dox/Mn-LTSLs during hyperthermia had a higher concentration of doxorubicin at the periphery of the tumor (i.e., near normalized radius 1.0) than near the heating catheter (i.e., near normalized radius 0) (Fig. 2, D and E). These six profiles were averaged to produce the “LTSL during HT” profile shown in Fig. 2, E. By contrast, rats that received Dox/Mn-LTSL before hyperthermia had higher concentrations of doxorubicin near the center of the tumor (i.e., near the heating catheter) than at the tumor periphery, and rats that received Dox/Mn-LTSL...
on a split-dose schedule had a relatively uniform concentration of doxorubicin throughout the tumor. The 95% confidence intervals indicated considerable intragroup heterogeneity. The radial drug distribution ratios (AUC ratios) were statistically significantly different in all three pairwise comparisons of the Dox/Mn-LTSL plus hyperthermia groups (LTSL during versus before hyperthermia: \( P = .003 \); LTSL during versus split-dose hyperthermia: \( P = .015 \); LTSL before versus split-dose hyperthermia: \( P = .010 \)) (Table 1).

### Total Amount and Concentration of Doxorubicin Delivered to the Tumor

We used MRI data to estimate the mean amount of doxorubicin that was delivered to the tumor and the mean initial rate of drug delivery for rats treated with each of the three Dox/Mn-LTSL plus hyperthermia protocols (\( n = 6 – 7 \) rats per group). The mean total amount of doxorubicin delivered to tumors of rats treated with Dox/Mn-LTSL during hyperthermia was 39.5 \( \mu \text{g} \) (Fig. 3, Table 1), which was equivalent to 5.5% of the injected dose (mean dose = 712 \( \mu \text{g} \)). Rats treated with Dox/Mn-LTSL before hyperthermia accumulated statistically significantly less doxorubicin in their tumors than rats treated with Dox/Mn-LTSL during hyperthermia (24.5 \( \mu \text{g} \), difference = 15.0 \( \mu \text{g} \), 95% CI = 3.9 to 26.1 \( \mu \text{g} \), \( P = .015 \); Fig. 3, Table 1). The split dose of LTSL resulted in an intermediate tumor doxorubicin level (33.5 \( \mu \text{g} \), 95% CI = 20.8 to 46.3 \( \mu \text{g} \)). We observed considerable heterogeneity in the final amount of doxorubicin among the tumors in each of the three Dox/Mn-LTSL plus hyperthermia treatment groups.

The initial rate of doxorubicin accumulation over the first 3 minutes was statistically significantly higher for Dox/Mn-LTSL administered during hyperthermia than for Dox/Mn-LTSL administered before hyperthermia (9.8 versus 1.8 \( \mu \text{g/min} \), difference = 8.0 \( \mu \text{g/min} \), 95% CI = 6.8 to 12.8 \( \mu \text{g/min} \), \( P = .003 \); Fig. 3, Table 1). (Note that the zero time point marks the magnetic resonance image acquired just before Dox/Mn-LTSL and hyperthermia were administered combined in the tumor. Rats that received LTSLs before hyperthermia [dashed line] or split dose [gray line] had intact liposomes circulating at this point, so the measured amount of doxorubicin was correspondingly greater than zero.)
from that of Dox/Mn-LTSL administered during hyperthermia. Rats treated with free doxorubicin during hyperthermia had a mean tumor doxorubicin concentration that was higher than rats treated with free doxorubicin alone (mean 6.0 versus 2.8 ng/mg, difference = 3.2 ng/mg, 95% CI = 1.9 to 4.5 ng/mg, P = .049; Fig. 4, A), as has been previously reported (17). Rats treated with Dox/Mn-LTSL alone did not show increased tumor uptake of doxorubicin compared with rats treated with free doxorubicin; however, the early time point (90 minutes after injection) does not reflect the peak concentration of sterically stabilized liposomes as they extravasate (18).

Free doxorubicin is known to cause cardiac damage that can lead to congestive heart failure, which limits the cumulative clinical dose to less than 500 mg/m² for most patients (46). Therefore, heart concentrations of doxorubicin were measured and compared among all treatment groups in this study to examine whether heart exposure was increased or decreased using LTSL versus free doxorubicin. The mean concentration of doxorubicin in rat heart was 9.9 ng/mg (95% CI = 8.6 to 11.2 ng/mg) for all treatment groups combined and did not differ statistically significantly between any of the treatment groups (data not shown).

**Antitumor Effect**

We next examined the effects of the various treatments on tumor growth by comparing the amount of time required for tumors to reach five times the volume that they were on the day of treatment (n = 6–7 rats per group). The rats in the Dox/Mn-LTSL plus hyperthermia groups were the same rats that were used for MRI analyses (Figs. 2 and 3, Table 1). The administration of Dox/Mn-LTSL during hyperthermia resulted in the greatest antitumor effect, with a median of 34 days (95% CI = 30 days to ∞) to five times the tumor volume on the day of treatment compared with 18.5 days (95% CI = 16 to 23 days) for Dox/Mn-LTSL administered before hyperthermia and 22.5 days (95% CI = 15 to 25 days) for the Dox/Mn-LTSL split dose (Fig. 4, B and C). The amount of time for the tumors to reach five times the volume at the start of treatment was statistically significantly longer for the group that received Dox/Mn-LTSL during hyperthermia than for all other groups except the Dox/Mn-LTSL split-dose group (Dox/Mn-LTSL during hyperthermia versus control or hyperthermia alone, P = .004; versus free doxorubicin, P = .004; versus free doxorubicin plus hyperthermia, P = .032; versus Dox/Mn-LTSL alone, P = .001; versus Dox/Mn-LTSL before hyperthermia, P = .007; versus Dox/Mn-LTSL split-dose hyperthermia, P = .071, Wilcoxon test). Free doxorubicin resulted in statistically significantly longer tumor growth times than the saline control, both with (P = .007) or without hyperthermia (P = .004), as has been previously observed in this doxorubicin-sensitive tumor model (19–22). Hyperthermia alone yielded minimal tumor growth delay, as did Dox/Mn-LTSL alone. Four rats showed complete tumor regression upon censoring at 60 days: two of the seven rats that received Dox/Mn-LTSL during hyperthermia, one of the six rats that received Dox/Mn-LTSL split dose, and one of the six rats that received free doxorubicin plus hyperthermia (Fig. 4, C).

**Tumor Drug Delivery and Antitumor Effect**

The scatter plot in Fig. 5 shows the relationship between tumor response (as measured by the time to five times the original tumor...
volume) and overall tumor doxorubicin concentration (as estimated from MRI data) in the three groups of rats that received Dox/Mn-LTSLs and hyperthermia. Rats that had the highest tumor doxorubicin concentrations showed the best tumor responses (i.e., the longest times to five times the initial tumor volume). Conversely, rats that had the lowest tumor doxorubicin concentrations (those receiving Dox/Mn-LTSL before hyperthermia) had the worst tumor responses (i.e., the shortest times to five times the initial tumor volume).

A Cox proportional hazards regression analysis of growth time versus tumor doxorubicin concentration among the three treatment groups resulted in an estimated regression parameter (b) of −0.19 (95% CI = −0.35 to −0.03, P = .023), indicating that MRI-based tumor doxorubicin concentration was statistically significantly associated with the time to five times the original tumor volume. It is notable that the scatter plot shows variable tumor growth times associated with the time to five times the original tumor volume. For all drug delivery systems, the pharmacokinetics and the biodistribution of the drug are identical to those of the carrier until the drug is released (1). LTSLs are unique because drug release occurs intravascularly, and accumulation of free doxorubicin in the tumor is dependent on the local rate of drug. In vitro studies have shown that Dox-LTSLs begin to release drug slowly at a temperature of 39 °C and exhibit a sharp increase in the rate of drug release at 41.3 °C (3,5). In this study, when liposomes were injected during steady-state hyperthermia, most of the tumor had achieved a temperature of 41.3 °C or higher (8). Therefore, the local rate of content release in the tumor was very high using this protocol (9.8 μg doxorubicin/min over the first 3 minutes). Conversely, when liposomes were administered before hyperthermia, the tumor did not reach steady-state temperatures until 30 minutes after injection (8). As the tumor heated to 41.3 °C, the local rate of liposomal drug release was relatively slow (1.8 μg doxorubicin/min over the first 3 minutes); thus, the liposomes may not have released 100% of their contents as they passed through the tumor. The slower local release rate, in addition to any systemic release of contents or liposome clearance, resulted in a lower final doxorubicin concentration in the tumor after administration of Dox/Mn-LTSL before hyperthermia when compared with that after Dox/Mn-LTSL administration during hyperthermia.

One limitation of this study was that the use of a centrally located heating catheter made it difficult to achieve identical temperature profiles in tumors of different shapes and sizes. Because the liposome release rate is very sensitive to temperature, the variability in temperature likely contributed to the observed intragroup heterogeneity in drug delivery and tumor response. For example, the size of the tumor shown in Fig. 2, A, was smaller than the average size of the tumors in that group of rats and in addition, the heating catheter was displaced toward the periphery. As a result, this tumor attained high peripheral temperatures and drug levels, resulting in complete regression. In the clinical setting, radiofrequency or microwave devices combined with noninvasive thermometry could be used to provide better control of local hyperthermia (24). In addition, because human tumors are much larger than rat tumors, these devices are capable of directing heat to specific regions of human tumors.

**Discussion**

Most systemic drug delivery systems, including commercially available liposomes, such as liposomal doxorubicin HCl and liposomal daunorubicin citrate, target tumors by extravasation from leaky tumor vessels followed by passive drug release into the tumor interstitium over the course of several days (1,18). Other liposome formulations also use specific antibodies or ligands to promote intracellular uptake of the carriers (23). By contrast, the temperature-sensitive liposomes used in this study exhibit rapid intravascular release at the permissive temperature, exposing tumor endothelial and perivascular cells to high concentrations of free drug (2).

Our MRI-based analyses of temporal and spatial drug distribution with Dox/Mn-LTSL plus hyperthermia suggest that tumor drug delivery patterns with this formulation are governed primarily by perfusion pattern and temperature. In the fibrosarcoma model, the main tumor arteries enter the tumor at its periphery (8). We found that intravenous injection of liposomes during hyperthermia resulted in rapid release of contents from the liposomes as they entered the tumor from this peripheral vascular source. Although the entire tumor was heated, the liposomes apparently released almost all contents before they reached the center of the tumor, yielding a peripheral doxorubicin distribution. Conversely, injection of liposomes before hyperthermia allowed the liposomes to perfuse throughout the tumor before releasing. When hyperthermia was initiated, liposome contents were released near the central heat source. In this case, the liposomes were apparently depleted of contents before the periphery was fully heated, yielding a central doxorubicin distribution. By splitting the dose (i.e., by injecting liposomes before and during hyperthermia), we achieved a more uniform drug distribution throughout the tumor. Spatial drug distribution could be controlled in a radial pattern with this tumor model because the perfusion source was peripheral and the heat source was central. Thus, we have demonstrated real-time drug dose “painting,” monitored by MRI and controlled by targeted local hyperthermia.

For all drug delivery systems, the pharmacokinetics and the biodistribution of the drug are identical to those of the carrier until the drug is released (1). LTSLs are unique because drug release occurs intravascularly, and accumulation of free doxorubicin in the tumor is dependent on the local rate of drug. In vitro studies have shown that Dox-LTSLs begin to release drug slowly at a temperature of 39 °C and exhibit a sharp increase in the rate of drug release at 41.3 °C (3,5). In this study, when liposomes were injected during steady-state hyperthermia, most of the tumor had achieved a temperature of 41.3 °C or higher (8). Therefore, the local rate of content release in the tumor was very high using this protocol (9.8 μg doxorubicin/min over the first 3 minutes). Conversely, when liposomes were administered before hyperthermia, the tumor did not reach steady-state temperatures until 30 minutes after injection (8). As the tumor heated to 41.3 °C, the local rate of liposomal drug release was relatively slow (1.8 μg doxorubicin/min over the first 3 minutes); thus, the liposomes may not have released 100% of their contents as they passed through the tumor. The slower local release rate, in addition to any systemic release of contents or liposome clearance, resulted in a lower final doxorubicin concentration in the tumor after administration of Dox/Mn-LTSL before hyperthermia when compared with that after Dox/Mn-LTSL administration during hyperthermia.

One limitation of this study was that the use of a centrally located heating catheter made it difficult to achieve identical temperature profiles in tumors of different shapes and sizes. Because the liposome release rate is very sensitive to temperature, the variability in temperature likely contributed to the observed intragroup heterogeneity in drug delivery and tumor response. For example, the size of the tumor shown in Fig. 2, A, was smaller than the average size of the tumors in that group of rats and in addition, the heating catheter was displaced toward the periphery. As a result, this tumor attained high peripheral temperatures and drug levels, resulting in complete regression. In the clinical setting, radiofrequency or microwave devices combined with noninvasive thermometry could be used to provide better control of local hyperthermia (24). In addition, because human tumors are much larger than rat tumors, these devices are capable of directing heat to specific regions of human tumors.
We found that injection of liposomes during hyperthermia resulted in the best tumor response compared with all other treatment protocols in this study. The improvement over injection before plus during hyperthermia (split dose) was likely related to the different patterns of drug accumulation in the tumor, whereas the improvement over other protocols was consistent with a faster accumulation rate and greater final concentration of doxorubicin in the tumor using LTSL during hyperthermia. Tumor drug levels (i.e., final concentrations at the end of therapy) are often used as predictors of chemotherapeutic outcome. In previously published studies in human tumor xenografts (2), the tumor doxorubicin concentrations resulting from various doxorubicin formulations (including Dox-LTSL) were tightly correlated with tumor growth time ($R^2 = .98$). Likewise, in this study, tumor doxorubicin concentration was statistically significantly associated with the time it took for tumors to reach five times the original tumor volume, and tumors with the highest doxorubicin levels displayed complete regression. Although drug concentration in tumors is often measured, the rate of drug accumulation in tumors has rarely been assessed or used to predict outcome. However, it is well known that a gradual (low rate) exposure of tumor cells to chemotherapeutic agents in vitro can select for mechanisms of resistance and survival (25). Conversely, the rapid rate of doxorubicin exposure achieved with LTSL during hyperthermia may be more likely to overwhelm these mechanisms.

We also found that the pattern of doxorubicin accumulation may influence antitumor efficacy. Specifically, rats treated with Dox/Mn-LTSL during hyperthermia, which resulted in peripheral drug distribution, showed longer tumor growth times than rats treated with Dox/Mn-LTSL split dose, which resulted in uniform drug distribution, even though the tumor doxorubicin concentrations did not differ statistically significantly between these groups. Among the individual rats, this discrepancy in tumor response was most apparent for tumors with intermediate tumor doxorubicin concentrations (13 to 17 ng/mg). Chen et al. (6) showed that tumors treated with Dox-LTSL during hyperthermia undergo vascular destruction. In the fibrosarcoma model used in this study, the vascular source is in the periphery of the tumor (8). Therefore, the perfusion-delimited peripheral distribution pattern we observed may have permitted doxorubicin to target the vessels that feed the tumor, thus achieving a substantial antivasular effect.

Our results indicate that Dox/Mn-LTSL in combination with local hyperthermia causes primarily intravascular drug release as opposed to interstitial drug release. It is well known that 100-nm liposomes (both LTSLs and non–temperature-sensitive liposomes [NTSLs]) extravasate in tumors, such that maximum tumor drug concentrations are reached several days after injection. Hyperthermia increases the rate of extravasation, resulting in maximum drug concentrations within hours (7,27,28). Our previous studies in a human squamous cell carcinoma xenograft model (FaDu) (2,3) showed that LTSLs with hyperthermia led to four- to fivefold higher tumor doxorubicin concentrations and better antitumor effects than comparably sized (100 nm) NTSLs with hyperthermia. Because the liposomes themselves (i.e., the drug carriers) should have exhibited similar extravasation and accumulation patterns, we reasoned that the higher drug concentration achieved with LTSL plus hyperthermia than with NTSL plus hyperthermia was due to intravascular release of free doxorubicin (2). This interpretation was supported by the rapid in vitro release kinetics of this formulation (4). This study further strengthens this argument by showing that the rate of drug accumulation in tumors treated with LTSL plus hyperthermia (10 minutes to maximum drug concentration) is much faster than the rate of hyperthermia-mediated extravasation. As in the FaDu studies (2,3), we also found that this intravascular release resulted in higher tumor drug concentrations and greater antitumor effects.

Results from multiple preclinical models suggest that vascular shutdown is part of the mechanism for the enhancement of antitumor effect using LTSL plus hyperthermia compared with free doxorubicin (2,3,6). The mouse FaDu xenograft studies described above (2,3) used a water bath to heat tumors, thus achieving more uniform temperatures and a more uniform drug distribution throughout the tumor compared with the hot water catheter heating method used in this rat fibrosarcoma study (2,8,29). Despite these differences, however, the evidence presented above suggests that intravascular drug release occurred in both models. This intravascular release exposes endothelial cells to the highest drug concentrations, which may lead to endothelial cell damage or death. Using dorsal skinfold window chamber models to test directly for antivasular effects, we found that LTSL plus hyperthermia leads to reduced red blood cell velocity and microvessel density in the FaDu tumor (6) and in a 4T1 mouse mammary carcinoma model (Chen Q, Tong S, Dewhirst MW, Yuan F: unpublished observations). These results argue for using a treatment schedule in which LTSLs are administered after thermal steady state has been reached, to expose the vasculature to the highest drug concentration. In future experiments, we will further investigate the antivasular effects of this therapy using a variety of functional imaging and pathologic methods.

The LTSLs that were used in the experiments reported here could also be used for local delivery of a variety of hydrophilic or amphipilic drugs. As emphasized in this study, the mechanism of action of a given formulation would depend on the hyperthermia schedule used as well as the activity of the free drug. Although our findings for LTSL during hyperthermia are consistent with intravascular drug release, previous studies have shown that in the absence of heat, LTSLs slowly extravasate in a manner similar to liposomal doxorubicin HCl (27). Therefore, it seems likely that interstitial drug release could be achieved with LTSLs by allowing the liposomes to extravasate before heating the tumor. It should also be noted that for intravascular targeted release with LTSL, the liposome size need not be limited to 100 nm because transvascular permeability is not required. Therefore, in future studies, a larger liposome could be used to deliver even greater amounts of drug.

This work represents the first longitudinal study of treatment response using a multimodal liposome for both MRI and therapy. This study is also unique in that we used the $T_1$-based method for calculating tumor doxorubicin concentrations for a quantitative (rather than qualitative) description of tumor drug delivery. Several groups have used positron emission tomography to evaluate therapeutic delivery of radiolabeled liposomes (30–33). However, this imaging method has limited spatial resolution and requires a cyclotron and specialized chemistry. Most magnetic resonance–imageable liposomal formulations have been developed
for diagnostic purposes rather than for drug delivery (34,35). Recently, Port et al. (36) showed that subcutaneous injection of a liposome encapsulating both drug (fludarabine) and magnetic resonance contrast agent resulted in passive release of contents. In addition, coinusions of imageable liposomes and liposomal doxorubicin have been used to study convection-enhanced drug delivery in rat glioma (37–40). A few imageable pH-sensitive and temperature-sensitive liposomes have been developed (41–45), but none have been used to observe triggered release within a tumor.

The most important technical limitation of our T1-based method for measuring tumor doxorubicin concentration is the requirement that doxorubicin and manganese remain colocalized after liposome release (8,9). Our analysis assumes complete colocalization, but this assumption does not take into account the tendency of doxorubicin to bind to proteins and DNA (46). However, our magnetic resonance movies (see Supplementary Movies 1–3) show a stable image for at least 30 minutes after therapy, suggesting that manganese is also retained in the specific tumor regions where it is released. Indeed, other studies have shown that free manganese tends to bind to tissues (47–49). Furthermore, we have previously demonstrated a strong linear correlation between T1-based tumor doxorubicin concentration and HPLC-based tumor doxorubicin concentration (slope = 0.86 ± 0.07 [standard error], intercept = 0.01 ± 1.45 ng/mg, n = 48 samples, mixed-effects linear regression model), even though tumors were harvested 45 minutes after liposome injection (9). In future studies, it may be possible to use this published correlation to estimate the HPLC-based doxorubicin concentration (i.e., the gold standard for determining tumor drug concentrations) from the MRI-based tumor drug concentration. Other technical limitations for our image analysis method include low signal-to-noise ratios at low manganese concentrations and extreme T1 shortening at high manganese concentrations (8), but the range of doxorubicin concentrations that can be estimated accurately (from 2 to 50 ng/mg) did not limit this study.

Overall, this study demonstrates that multimodal liposomes containing both drug and contrast agent such as Dox/Mn-LTLSs permit real-time evaluation of therapeutic protocols in association with outcome on an individual subject basis. Furthermore, the rapid intravascular release that is unique to this formulation facilitates control of drug distribution (i.e., drug dose painting) through perfusion- or temperature-limited delivery. Most importantly, these data suggest that the optimal scheduling of Dox-LTLS therapy is to administer liposomes during steady-state local hyperthermia.

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Notes

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