A Tracer Dose of Technetium-99m–Labeled Liposomes Can Estimate the Effect of Hyperthermia on Intratumoral Doxil Extravasation

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Abstract

Purpose: A noninvasive method to monitor intratumoral Doxil delivery in individual patients during targeted tumor therapy is important to predict treatment response. The purpose of this study was to determine if a small tracer dose of technetium-99m (^{99m}Tc)–labeled liposomes could be used to quantify the effect of local hyperthermia on intratumoral Doxil extravasation.

Experimental Design: Experiments were carried out in a rat fibrosarcoma model with transplanted thigh tumors. Liposomes of approximately same size and composition as Doxil were radiolabeled using [technetium-99m (^{99m}Tc)]exametazime. Eight treatment groups received either Doxil, a tracer dose or a large dose of ^{99m}Tc-labeled liposomes, or a combination of tracer and Doxil, with or without hyperthermia. This design was chosen to assure that coadministration of both liposomal formulations did not influence their intratumoral distribution. Hyperthermia was done for 45 minutes. Scintigraphic images were obtained at 5 and 18 hours. At 18 hours, tumors were removed and gamma counts as well as doxorubicin concentrations were measured.

Results: Intratumoral extravasation of the ^{99m}Tc-labeled tracer could be imaged scintigraphically under normothermic and hyperthermic conditions. The thermal enhancement ratio was slightly higher for radiolabeled liposomes than for doxorubicin concentration. However, there was a significant positive correlation of intratumoral doxorubicin concentration and intratumoral uptake of the radiolabeled tracer (expressed as percentage of the injected dose per gram of tissue). Coadministration of radiolabeled liposomes did not negatively influence the amount of drug delivered with Doxil.

Conclusions: The use of a radiolabeled tracer has potential value to monitor drug delivery and estimate the effect of an intervention aimed to increase liposomal accumulation, such as local hyperthermia.

Liposomes are small phospholipid vesicles that have achieved wide interest as carrier systems for targeted delivery of chemotherapeutic drugs (1–5). The development of polyethyleneglycol coating, the so-called pegylated or STEALTH liposomes, was important as it formed the basis for liposomes escaping early recognition and phagocytosis by the reticuloendothelial system (6). Pegylated liposomes have long circulation times, permitting passive transvascular accumulation with increased drug concentrations in solid tumors (6, 7). Part of the mechanism for enhanced liposome accumulation achieved with pegylation comes from higher vascular permeability in solid tumors (8).

Doxil, a pegylated liposomal formulation of doxorubicin, has been investigated most intensively. Doxil was first approved by the Federal Drug Administration in 1995 for treatment of Kaposi sarcoma (9). Several clinical trials have been conducted with Doxil for treatment of ovarian cancer (10, 11); advanced breast cancer (12, 13); and several other cancers, such as sarcoma (14), pediatric solid tumors (15), advanced leiomyosarcoma (16), lung cancer, and multiple myeloma (17, 18).

Doxorubicin, a cytotoxic anthracycline antibiotic, is entrapped in the internal aqueous phase of the liposome via an ammonium sulfate chemical gradient in Doxil's formulation. This gradient facilitates high doxorubicin loading into the liposome with a high drug/lipid ratio. The concentration of doxorubicin is 2 mg/mL and the lipid content of Doxil is ~16 mg/mL, yielding a mean vesicle size of ~90 nm. The single lipid bilayer is made

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of hydrogenated soy phosphatidylcholine, cholesterol, and distearoyl phosphatidylethalomine, which serves as an anchor for polyethyleneglycol (DSPE-PEG_{2000}; ref. 19).

Pharmacokinetically, the area under the doxorubicin concentration-time curve is increased at least 60-fold with Doxil compared with free doxorubicin. Doxorubicin is released effectively after liposomal extravasation due to the difference in kinetics between its diffusion out of the liposome and its accumulation rate within the tumor, yielding tumor peak concentrations occurring up to 48 hours postdelivery (19).

Hyperthermia augments intratumoral accumulation of liposomes over that achieved by passive accumulation. Local hyperthermia increases tumor perfusion and further opens the permeable tumor microvasculature, resulting in significantly increased liposomal delivery (5) and extravasation (8, 20–23). Normal tissues are resistant to moderate thermal effects on vascular permeability (temperature <44°C; refs. 21, 22). It has been demonstrated that temperatures of 40°C to 42°C are sufficient to increase tumor vascular permeability, with maximal extravasation rates occurring at 42°C, without damage to the normal vasculature (20). These relatively mild hyperthermic temperatures are achievable in the clinical setting.

Hyperthermic enhancement of tumor liposomal accumulation is greatest with 100-nm liposomes, compared with liposomes in the 200 to 400 nm size range (21). Doxil liposomes, with an average diameter of 90 nm, have the ideal size to maximize the effect achieved by combining them with hyperthermia (21). Several studies have shown that hyperthermia enhances intratumoral drug uptake and cytotoxic effects compared with those of liposomal drug alone or hyperthermia given with free drug (21–24).

Intratumoral drug concentrations are one of the best predictors of treatment outcome (1). Thus, a noninvasive technique that could accurately quantify the magnitude of drug within an individual tumor, both with and without hyperthermia, is highly desirable and would find broad clinical application. Estimation of the expected intratumoral drug concentration might permit the selection of patients who are most likely to benefit from the combination of hyperthermia and drug-containing liposomes. Scintigraphic imaging is a valuable tool for this purpose. It provides the ability to track and quantify the distribution of liposomes in the body noninvasively using a gamma-emitting radionuclide label (3, 5, 25). A stable labeling method for ^{99m}Tc is well established using glutathione-containing liposomes with exametazime (hexamethylpropyleneamine; refs. 25, 26).

In prior studies, Matteucci et al. (5) showed that ^{99m}Tc-labeled liposomes could be used to monitor increased liposomal uptake after local hyperthermia in cats with spontaneous soft tissue sarcomas. In that study, the radiolabeled liposomes were given alone and nondoxorubicin-containing liposomes were administered.

What is needed in a setting of hyperthermia and drug-containing liposomes is a way to monitor the effect of hyperthermia on liposome extravasation without having to biopsy the tumor. In this study, the objective was to determine if a small tracer dose of ^{99m}Tc-labeled liposomes administered concomitantly with size-matched therapeutic doxorubicin-containing liposomes could accurately estimate the effect of hyperthermia on intratumoral doxorubicin concentration. A secondary aim was to assure that coadministration of radiolabeled liposomes did not negatively influence intratumoral extravasation of the drug-carrying liposomes.

Materials and Methods

Animal model

All experiments were carried out in female Fisher 344 rats weighing ~160 g (Charles River Laboratories, Raleigh, NC). Rats were injected s.c. in the left thigh region with a transplantable fibrosarcoma cell line (MCA-R; ref. 27). This tumor line was originally developed by s.c. injection of methylcholanthrene into Fisher 344 rats and is maintained by serial transplantation. Tumors were allowed to grow until they were ~15 mm in diameter. Animals were kept in standard housing with regular light and dark cycles. The animal protocol was approved by the Institutional Animal Care and Use Committees of Duke University and North Carolina State University.

Experiments

A total of eight experimental groups with seven rats per group were used, and experiments were conducted in two cohorts. The first cohort consisted of two groups: large ^{99m}Tc-liposome dose with and without heat. The second cohort consisted of the following six groups: Doxil with and without heat, tracer ^{99m}Tc-liposome dose with and without heat, and tracer ^{99m}Tc-liposome dose plus Doxil with and without heat (Table 1).

Table 1. Results of ^{99m}Tc imaging and quantification, and intratumoral doxorubicin concentration, by treatment group (n = 7)

Group	Treatment	TTR_{18hr}, mean (SD)	TER_{18hr}	P*	%ID/g, mean (SD)	TER_{18hr}	P	Dox (ng/mg), mean (SD)	TER_{18hr}	P*
1	L	3.4 (1.3)	3.5	0.0006	—			—		
2	L+H	11.7 (2.9)			—			—		
3	D	—			—			8.8 (1.7)	2.6	0.0006
4	D+H	—			—			22.4 (6.7)		
5	T	2.3 (0.7)	3.9	0.0006	0.6 (0.2)	4.0	0.006	—		
6	T+H	9.0 (3.9)			2.3 (1.2)			—		
7	D+T	2.5 (0.4)	4.4	0.0006	0.6 (0.3)	4.3	0.006	9.3 (4.7)	3.0	0.0023
8	D+T+H	11.0 (2.4)			2.6 (0.6)			27.3 (10.5)		

Abbreviations: TTR_{18hr}, scintigraphic tumor-to-thigh (background) ratio at 18 hours; TER_{18hr}, thermal enhancement ratio at 18 hours; Dox, doxorubicin; L, large dose of radiolabeled liposomes equal in lipid content to a dose of Doxil; H, hyperthermia; D, Doxil; T, tracer dose of radiolabeled liposomes.
*Wilcoxon P values for comparing nonhyperthermic group with hyperthermic group.

The large dose of radiolabeled liposomes simulated the equivalent lipid amount that would be administered with Doxil. The lipid dose of the radiolabeled tracer was 10% of the Doxil lipid dose.

Doxil was administered at a dose of 45 mg/m^2. The Doxil formulation contains 8 mg lipid/mg doxorubicin. A body-surface-area ratio factor of 5.9 kg/m^2 was used for the rats. Thus, for a 160 g rat, a standard Doxil dose would be equal to 61 mg lipid per kilogram or 9.6 mg lipid (0.6 mL). Liposomes were injected via a tail vein catheter. In the first cohort, liposomes were radiolabeled with 0.15 mCi ^{99m}Tc per milligram of lipid, and a total lipid dose equivalent to Doxil was injected (9.6 mg). In the second cohort, the small tracer dose was labeled with 0.45 mCi ^{99m}Tc per milligram of lipid, and 1 mg of the lipid was administered, i.e., ~10% of the 9.6 mg lipid dose mentioned above.

In rats receiving hyperthermia, liposomes were injected at the beginning of a 45-minute heating period as soon as a steady-state intratumoral temperature was achieved (28). Scintigraphic imaging was done at 5 and 18 hours after injection. The 18-hour time point was chosen to allow liposomes to accumulate in the tumor while still giving a reasonable 99mTc signal. This time point has also been used in our previous feline study (5). The earlier 5-hour time point was chosen for some comparison. At 18 hours, tumors were removed and total tumor activity was measured. Tumors were then frozen at −80°C to allow for radioactive decay, after which the samples were subjected to high-performance liquid chromatography (HPLC) analysis to determine intratumoral doxorubicin concentration.

Liposome materials and preparation

Doxil (Ortho Biotech Products, Raritan, NJ) was used as the pegylated nonradioactive liposomal doxorubicin formulation. For the radiolabeled liposomes, reduced glutathione–loaded 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC)/cholesterol/polyethyleneglycol derivative of distearoyl phosphatidylethalomine (DSPE-PEG$_{2000}$) liposomes were purchased from the British Columbia Cancer Agency.

The liposome preparation and reduced glutathione loading were done as previously described (5). Briefly, unilamellar vesicles were prepared from 1,2-distearoyl-*sn*-glycero-3-phosphocholine/cholesterol/DSPE-PEG$_{2000}$ (ratio 75:50:3) by the film hydration method. Lipids at the indicated ratios were dissolved in chloroform and the solvent was evaporated under vacuum with a stream of nitrogen gas to remove all organic solvent. The resulting film was then hydrated with 200 mmol reduced glutathione in Dulbecco's PBS (pH 7.40) at 58°C to 60°C with constant stirring. The formed multilamellar vesicles were then extruded through a thermobarrel extruder (Lipex Biomembranes, Vancouver, British Columbia, Canada), 100-nm pore filter, with ~300 to 400 p.s.i. pressure. Untrapped residual reduced glutathione was removed by passing the liposome suspension through a Sephadex G-50 column. The preformed reduced glutathione liposomes had a lipid concentration of 42.5 mg/mL solution and a mean liposome size of 137.5 nm.

Radiolabeling

For encapsulation of ^{99m}Tc into the aqueous phase of the preformed liposomes, the glutathione-exametazime (formerly hexamethylpropyleneamine) method was used. Briefly, ^{99m}Tc-exametazime was prepared using the Ceretec labeling kit (Amersham Health, Princeton, NJ), which contains 0.5 mg exametazime and 7.6 μg stannous chloride dehydrate. The kit was reconstituted with 3 mCi sodium pertechnetate ($^{99m}TcO_4$) in 0.77 mL of 0.9% NaCl solution (cohort 1) or with 7.2mCi $^{99m}TcO_4$ in 0.65 mL 0.9% NaCl (cohort 2) at room temperature for 5 minutes. The ^{99m}Tc-exametazime complex was then mixed with 0.43 or 0.35 mL preformed liposomes and incubated at room temperature with intermittent vortexing for 30 minutes. Labeling efficiency was determined by passing a 0.2-mL aliquot through a Sephadex G-50 column and then calculated as the percentage of the total radioactivity associated with the liposome fraction.

Heating method

Local hyperthermia was done under general anesthesia using isoflurane. Tumors were heated with thermal conduction by circulating warm water through a 16G catheter that was placed through the center of the tumor. The temperature of the heated water was ~50°C and the temperature of the outer surface of the catheter is 6°C cooler (44°C) than the heated water. A heat lamp was placed above the tumor to maintain the skin temperature between 36°C and 39°C. The core temperature of the rat was maintained by a rectal temperature–regulated heating pad. Hyperthermia was done for 45 minutes after reaching steady-state water temperature, which usually required 10 to 15 minutes.

With this approach, there is a temperature gradient from the center of the tumor (44°C) to the periphery (>39°C). This method and its temperature calibration profile have been previously described for the same tumor model and tumor size and the calibration data were applied to these experiments (28). The regimen ensures that the entire treated volume is in the temperature range to permit enhanced liposome extravasation (39-44°C; ref. 20).

We have previously reported that there is temperature dependence of liposome extravasation in this temperature range (20). Thus, we would expect that there might be spatial variation in the degree of extravasation. Nevertheless, the entire tumor should be sufficiently hot to permit some extravasation throughout the tumor volume. We have previously shown that this is the case by using magnetic resonance–imageable liposomes, where better spatial resolution is possible (28).

Nuclear imaging studies

Five and 18 hours after liposome injection, rats were anesthetized with isoflurane and placed in a sternal recumbency on a gamma camera equipped with a low-energy, all-purpose parallel-hole collimator that peaked at 140 keV. Planar whole-body scintigrams were recorded to quantify radiolabeled liposome distribution. The gamma camera was interfaced to a dedicated computer with nuclear medicine software. Static frame mode images were acquired (100,000 counts per image or 15-minute acquisition time) and stored with a digital matrix size of 256 × 256.

Regions of interest were drawn around the tumor and the comparable area in the contralateral thigh (same number of pixels) to correct for background. The same region of interest was used for both the 5- and 18-hour time points. The tumor-to-background (opposite thigh) ratio was calculated by dividing the mean counts per pixel in the tumor region of interest by the mean counts per pixel in the contralateral thigh region of interest. Thermal enhancement ratio was calculated by dividing the tumor-to-thigh ratio in the heated groups by the tumor-to-thigh ratio in the corresponding normothermic groups (Table 1).

Tumor gamma counts

In the second cohort of rats, the radioactivity of the dissected tumors was also determined in a well-type scintillation gamma counter and expressed as the percentage of the injected dose per gram of tissue (%ID/g). The dissected tumors were washed with saline, dried between folds of paper towel, and transferred to preweighed tubes. Four 5-μL-aliquot samples of injected liposomes served as controls. Thermal enhancement ratios were calculated by dividing the percentage of the injected dose per gram of tissue achieved with hyperthermia by the corresponding no-heat values (Table 1).

Intratumoral doxorubicin measurements

Materials. Doxorubicin, daunorubicin, and ammonium formate were from Sigma-Aldrich (St. Louis, MO). A Pure Flow, Inc. (Mebane, NC), system was used to obtain deionized water. HPLC-grade methanol, chloroform, and isopropanol were from Mallinckrodt Baker, Inc. (Phillipsburg, NJ) $AgNO_3$ was from Sigma Chemical Co. (St. Louis, MO).

Sample preparation. Tumor tissue was weighed, pulverized by a cryocrusher (cooled in ethanol/dry ice mixture), and transferred into 50-mL polypropylene tubes. An appropriate volume of water [volume (weight) of H_2O = 3 × weight of tissue] was added and the sample was homogenized by a rotor-stator homogenizer. The sample was aliquoted by adding 100 μL of the homogenate into a 2-mL polypropylene screw-capped vial and stored at −80°C. On the day of analysis, frozen tumor tissue homogenate (100 μL) was thawed and two new 1/4-in. ceramic beads (Q-BIOgene, Carlsbad, CA), 150 μL H_2O, 50 μL 33% $AgNO_3$, and 1.5 mL chloroform/isopropanol (2:1, v/v) were added. The sample was kept on ice bath for 10 minutes followed by a rapid homogenization in a FastPrep, model FP120, instrument (Q-BIOgene) at speed setting 4 for 30 seconds; this 10-minute cooling and homogenization cycle was repeated two more times followed by centrifugation at 4°C, 10,621 × *g* (10,000 rpm on Eppendorf 5408R centrifuge) for 3 minutes. The organic phase was drawn out using 3 mL all-plastic polypropylene syringe equipped with a 221/2 G needle and, after replacing the needle with a 0.45 μm nylon filter (National Scientific Company, Rockwood, TN), transferred into a 12 × 75 borosilicate glass test tube and evaporated to dryness by nitrogen. The sample was reconstituted by adding 200 μL HPLC mobile phase (see below) to the glass tube, vortexed for 10 seconds, sonicated for 10 minutes, and transferred into a 1.5 mL polypropylene microcentrifuge tube. The tube was centrifuged at 4°C, 10,621 × *g* for 5 minutes; 150 μL of the supernatant were drawn into a new microcentrifuge tube and centrifuged for another 5 minutes. One hundred microliters of the supernatant were taken into a new microcentrifuge tube and 20 μL of internal standard solution (1 μg/mL daunorubicin in methanol) were added. The tube was vortexed for 30 seconds. The solution was transferred into plastic injection vial and run on HPLC.

HPLC analysis. Total doxorubicin in tumor tissue was determined using a Waters 2695 HPLC system, Waters 474 Scanning Fluoresence Detector (excitation 480 nm, emission 550 nm, gain 100). Chromatography was done on a Waters column (Nova-Pak C_{18}, 3.9 × 150 mm) equipped with a guard column (Phenomenex "Security Guard" C_{18} cartridge, 4 mm L × 3 mm ID) and precolumn filter (Waters cartridge). Experimental conditions were as follows. Mobile phase A: 20 mmol/L formate buffer (pH 3.5). Mobile phase B: 100% methanol. Programmed flow: 0 to 8 minutes isocratic 55% A and 45% B; 8 to 11 minutes gradient from 55% A, 45% B to 30% A, 70% B; 11 to 15 minutes gradient from 30% A, 70% B to 55% A, 45% B. Flow rate: 1.5 mL/min. Typical back-pressure: 2,500 p.s.i. Column temperature: +50°C. Autoinjector sample compartment temperature: +4°C. Injection volume: 25 μL. Clean chromatograms were obtained with doxorubicin and daunorubicin (internal standard) retention times of 3.9 and 5.8 minutes, respectively. Standard curves (10 solutions, 0.0-10 μg/mL) were better than r^2 = 0.995.

Statistical considerations

Study design. This study was designed to investigate the feasibility of using a radiolabeled liposome tracer to estimate intratumoral Doxil concentration, with or without hyperthermia.

A sample size of seven rats per group was chosen to ensure sufficient pairwise group comparison power to detect a 3-fold increase in mean intratumoral extravasation or Doxil concentration for any hyperthermic group versus its corresponding nonhyperthermic group, with a significance level of 0.05 and consideration given to possibly up to 20% reduction in the mean intratumoral extravasation or Doxil concentration when a tracer dose of liposomes was coadministered together with Doxil. Randomization was carried out within each cohort, with rats being randomly allocated with equal probability to each of the experimental groups. Experiments in cohort 1 were conducted first to assure that the group size used to detect an effect of hyperthermia on liposome extravasation was adequate.

Data analysis. Descriptive summary statistics as well as nonparametric and parametric hypothesis testing results were reported for all and treatment-related variables, respectively, when appropriate. Pairwise comparisons of interest were done using Wilcoxon as well as *t* test/*F* test to guard against issues associated with the small group sample size. The linear regression method was used to explore the underlying relationships between the experimental factors and intratumoral liposome extravasation effect and/or Doxil concentration in tumor. Correlation analysis was carried out using the Pearson and Spearman correlation coefficients. Covariate adjustment was not considered in these regression analyses due to the small group sample size. Not all results were listed in this report. A *P* value of 0.05 was considered statistically significant. Multiple comparisons were not taken into account when calculating the *P* values.

Results

Fifty-six female rats with a median weight of 160 g (range 125-182 g, interquartile range 152-168 g) and median tumor volume of 1.76 cm^3 (range 0.46-5.66 cm^3, interquartile range 1.43-2.28 cm^3) were used in this study.

Hyperthermic effect

Tumor-to-background (thigh) ratio at 5 and 18 hours. Intratumoral accumulation of radiolabeled liposomes was detectable in all studies at both 5 and 18 hours. Qualitatively, higher uptake was observed in the heated groups, particularly at the 18-hour time point (Fig. 1). Hyperthermia caused a significant increase in intratumoral liposome accumulation in all hyperthermic groups compared with their nonheated control groups. The tumor-to-background ratio increased from the 5- to 18-hour time point. The greater accumulation at 18 hours could be visualized qualitatively in the heated groups, but was less obvious in the nonheated groups (data not shown). The thermal enhancement ratio ranged from 3.0 to 4.1 at 5 hours and 3.5 to 4.4 at 18 hours. Values at 18 hours were used for statistical comparisons, as no 5-hour values were available for percentage of the injected dose and doxorubicin concentration (Fig. 2; Table 1).

Percentage of injected dose per gram of tissue. In the second series of experiments, thermal enhancement of liposomal accumulation was also observed, as assessed by quantification of tumor radioactivity in a gamma counter. The thermal enhancement ratio based on the percentage of the injected dose per gram of tissue ranged from 4.0 to 4.3 for the radiolabeled tracer and the tracer plus Doxil groups and matched well with the thermal enhancement measured scintigraphically (Table 1).

Intratumoral doxorubicin concentration. The intratumoral doxorubicin concentration was also significantly higher in the two hyperthermic groups receiving Doxil comparison with their nonheated controls. The thermal enhancement ratio ranged between 2.6 and 3.0. This was slightly lower than the thermal enhancement ratio measured for the radiolabeled liposomes, assessed either by scintigraphy or gamma counter (Fig. 2; Table 1).

Correlation between radiolabeled tracer and doxorubicin concentration

A significant positive correlation was found between tracer uptake (expressed as %ID/g) and doxorubicin concentration in the nonheated and hyperthermic groups. This positive correlation was present whether control and heated groups

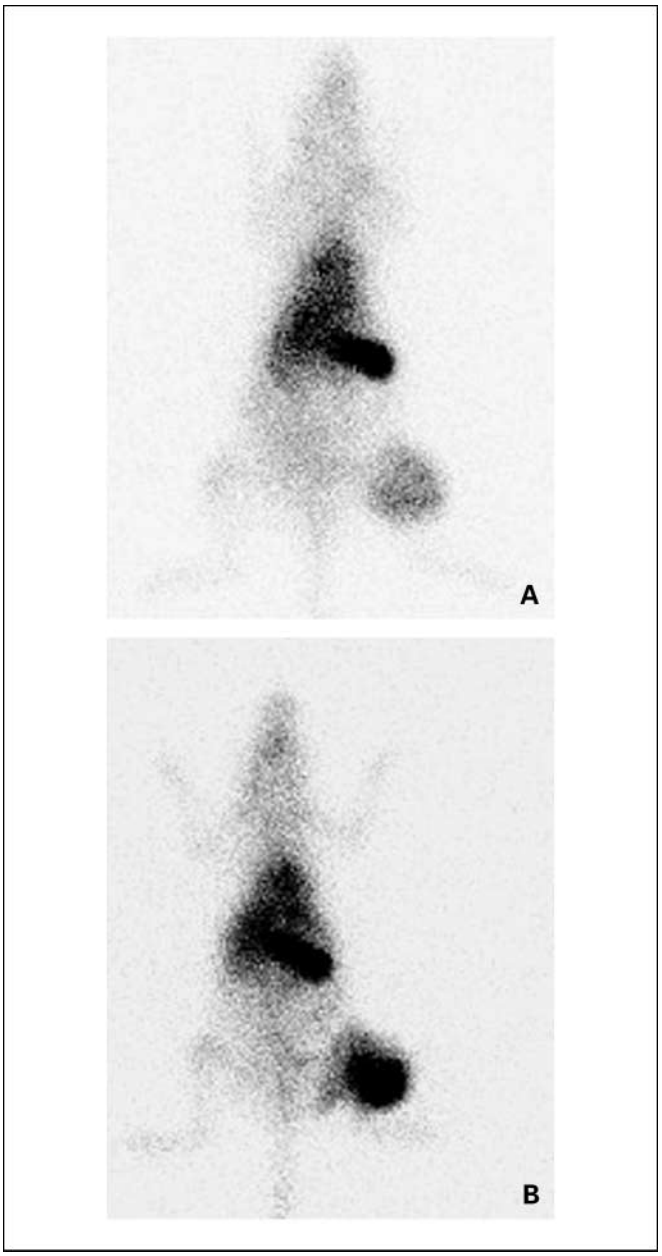

Fig. 1. Scintigraphic images of two rats treated without hyperthermia (*A*) or with local hyperthermia (*B*) 18 hours after injection of radiolabeled liposomes. Increased intratumoral accumulation of radiolabeled liposomes can be clearly seen as a result of hyperthermia. Subjectively, heart, spleen, and liver uptake is evident, likely reflecting persisting systemic circulation and endocytosis of liposomes.

were examined independently or combined. For the no-heat Doxil plus tracer dose group, the Pearson correlation coefficient is 0.89 (95% confidence interval, 0.41-0.98); for the heat Doxil plus tracer dose group, the Pearson correlation coefficient is 0.86 (95% confidence interval, 0.29-0.98); for both groups combined, the Pearson correlation coefficient is 0.92 (95% confidence interval, 0.77-0.98). The Spearman correlation analysis yielded similar results (data not shown). Furthermore, there was a statistically significant linear relationship between tracer uptake (expressed as %ID/g) and doxorubicin concentrations (Fig. 3).

Effect of liposome dose or coadminstration of radiolabeled tracer and Doxil

There was no evidence that intratumoral accumulation of radiolabeled liposomes was affected by liposome quantity or coadministration of Doxil under normothermic or hyperthermic conditions. This is noted by the similarity of tumor-to-thigh ratios and the percentage of injected dose per gram of tissue for groups 1, 5, and 7 as well as for groups 2, 6, and 8 (Fig. 2; Table 1). Pairwise 95% confidence intervals all included 0 (data not shown), indicating that the present study provided no evidence that liposome number or coadministration of Doxil under normothermic or hyperthermic conditions significantly affected the intratumoral accumulation of radiolabeled liposomes.

In addition, Doxil extravasation was not affected by the presence of tracer liposomes. Doxorubicin concentrations in the normothermic groups 3 and 7 and the hyperthermic groups 4 and 8 were not significantly different from each other at a nominal level of 0.05 (Fig. 2; Table 1).

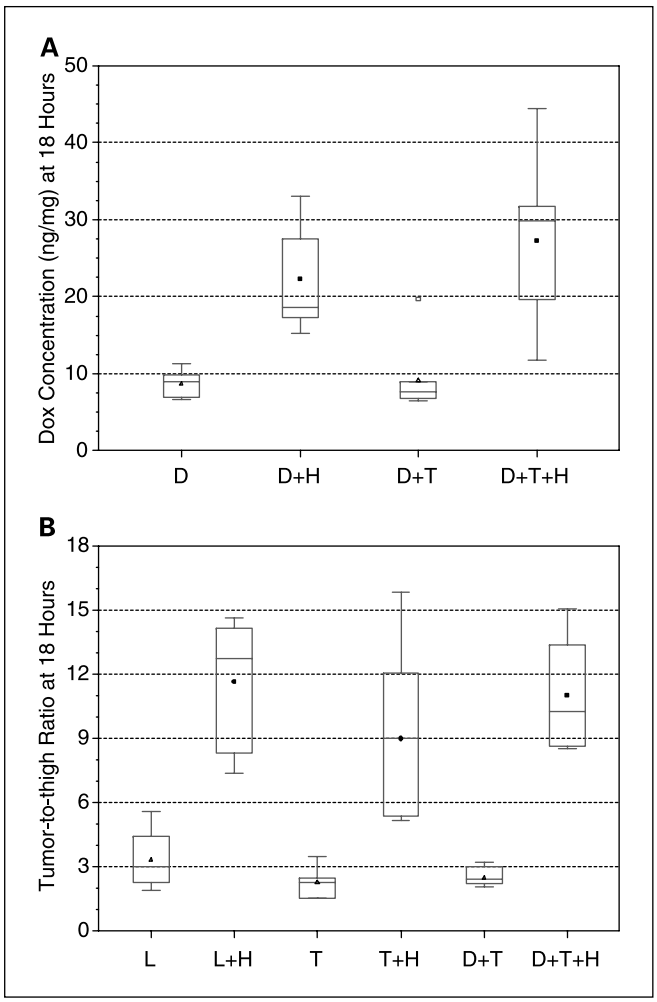

Fig. 2. Doxorubicin (*Dox*) concentrations (*A*) and tumor-to-thigh ratios (*B*) without or with hyperthermia at 18 hours in the different treatment groups. Neither number of injected ^{99m}Tc liposomes nor coadministration of radiolabeled tracer and Doxil significantly influenced their intratumoral extravasation under normothermic or hyperthermic conditions. Further, the significant increase of intratumoral doxorubicin or ^{99m}Tc liposomes after hyperthermia can be seen. *D,* doxil liposome; *H,* hyperthemia; *L,* large dose of radiolabeled liposomes; *T,* tracer dose of radiolabeled liposomes.

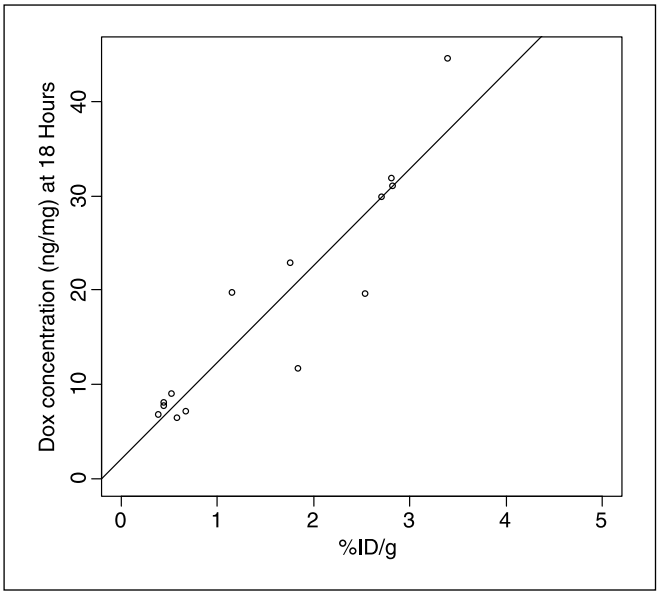

Fig. 3. Intratumoral doxorubicin concentration 18 hours postinjection as a function of intratumoral uptake of a ^{99m}Tc liposomal tracer (%ID/g). A significant positive correlation is present. This positive correlation was present whether control and heated groups were examined independently or combined. Combined data set. The Pearson correlation coefficient is 0.92 (95% confidence interval, 0.77-0.98). The linear regression line in the plot is $y = 2.05 + 10.28x$, where $R^2 = 0.86$; 95% confidence intervals for the intercept and slope are −2.89 to 6.98 and 7.69 to 12.87, respectively.

Discussion

In these studies, we have shown that delivery and accumulation of a small tracer dose of radiolabeled liposomes within a tumor can be imaged scintigraphically under normothermic and hyperthermic conditions. Furthermore, this tracer can be used to monitor the increased intratumoral extravasation of radiolabeled liposomes after intervention with local hyperthermia.

In previous work by our group (5), we showed the general feasibility of using radiolabeled liposomes to monitor thermal enhancement of liposomal uptake in spontaneous feline tumors. In that study, the radiolabeled liposomes were given in a large dose and no doxorubicin-containing liposomes were administered. We expanded this approach by confirming the validity of this technique with addition of a small lipid dose to be used as a radioactive tracer. The lipid dose of the tracer composed only 10% of the lipid amount that is administered with a conventional dose of Doxil.

Our thermal enhancement ratios of 3.5 to 4.4 are comparable with the previous study where a thermal enhancement ratio of 2 to 4 was found in most feline tumors (5). Therefore, the rat sarcoma model seems to be representative of spontaneously arising fibrosarcomas from a liposome transport and thermal conductivity standpoint. In terms of percentage of injected dose per gram of tumor tissue, as measured by gamma counter, the same magnitude of thermal enhancement was noticed.

This 3- to 4-fold increase in drug uptake is anticipated to have therapeutic benefits. We previously published results showing that hyperthermia increases liposomal uptake in a xenograft model by a factor of 4 to 5, using a Doxil-like liposome. The increase in drug concentration was associated with significant enhancement in growth delay, compared with controls, including free doxorubicin (29). There are several other published preclinical studies illustrating that a 2- to 4-fold increase in liposomal drug delivery results in enhancement in antitumor effect. This has been shown for several types of drugs, including cisplatinum and doxorubicin (22).

The percentage of injected dose of liposomes in the tumors was relatively low under normothermic conditions (~0.6%ID/g) compared with several other studies (30–32). This difference could be, in part, due to the use of human xenograft models in previous studies. We used a transplantable rat fibrosarcoma cell line (MCA-R) that was originally induced by s.c. injection of methylcholanthrene (27). In another murine study (MNU-induced breast cancer rat model), a similar low normothermic uptake was found, i.e., ~0.2%ID/g (2).

Tumor-to-thigh (background) signals were slightly higher at 18 hours compared with 5 hours. The increased accumulation of liposomes into areas of leaky vasculature with time is a well-known observation. This effect is explained by the enhanced permeability and retention effect, improving imaging signal as well as therapeutic drug concentrations (2, 19, 22). The effects of hyperthermia on liposome extravasation has been shown to persist several hours after heating (20).

Other studies suggested labeling therapeutic liposomes directly and thus directly tracking the drug-containing carrier (4, 33). Direct labeling of Caelyx with [^{99m}Tc]diethylenetriaminepentaacetic acid has been attempted in patients with glioblastoma and metastatic brain lesions (33). Laverman et al. (34) has since questioned this labeling technique, arguing that the method would result in an unstable radiolabeled product without a driving force that will facilitate the hydrophilic [^{99m}Tc]diethylenetriaminepentaacetic acid to pass the liposomal membrane and become trapped inside the liposome.

Bao et al. (4) used yet another labeling method: They labeled Doxil directly with [^{99m}Tc]*N*,*N*-bis(2-mercaptoethyl)-*N*′,*N*′-diethyl-ethylenediamine, and used the formulation in rats. The [^{99m}Tc]*N*,*N*-bis(2-mercaptoethyl)-*N*′,*N*′-diethyl-ethylenediamine complex is neutral and has a certain lipophilicity at a higher pH. It contains amine groups so it can be trapped inside the liposomes using an ammonium gradient. This mechanism enables direct radiolabeling of Doxil with the complex (4). Further studies in tumor-bearing animals will be of interest to evaluate this method. A disadvantage of the use of directly radiolabeled Doxil is its applicability in a clinical setting: Clinical use of such a drug delivery modality would require additional Food and Drug Administration approval as a modified drug.

Use of a radiotracer dose, as examined in this study, would still require regulatory approval, but it would be independent of the drug. For a purely radiolabeled liposome, the main regulatory point would be the radiation dose delivered with the tracer. The biodistribution of ^{99m}Tc-labeled liposomes has been studied in rats (35). Based on these studies, the critical organ receiving most radiation dose per unit of administered radiotracer is the spleen. A human patient administered a 20 mCi dose of ^{99m}Tc liposomes would receive an effective dose equivalent of ~0.7 rem, which is well within the 3 rem maximum allowed by the Food and Drug Administration.

An important requirement for our indirect tracer approach was that the tracer liposomes should not negatively affect the behavior of the drug-carrying liposomes, or vice versa. We

found no effect of one liposome type on the other with regard to liposome uptake into tumors. This is an important finding to move the tracer strategy forward into a clinical setting.

There are several studies that have evaluated radiolabeled liposomes for diagnostic purposes, including attempts of tracking lymphatic drainage and improving tumor-targeting systems (2, 4, 36–39). However, little is known about the feasibility of using radiolabeled liposomes to estimate intratumoral drug uptake or to quantify the effects of a therapeutic intervention, such as hyperthermia to increase liposomal drug accumulation (5, 33). The expanding use of Doxil in cancer chemotherapy makes the development of a reliable method to noninvasively monitor intratumoral liposomal drug uptake, with or without additional intervention, increasingly more desirable.

In this study, we investigated the feasibility of using a radiolabeled tracer to estimate intratumoral Doxil extravasation. We compared overall intratumoral radioactivity with overall drug concentration. We did not measure intracellular doxorubicin. However, we have done this previously, following administration of Doxil-like liposomes, with and without hyperthermia (29). In this prior work, we showed that hyperthermia treatment increased overall drug concentrations in tumor after liposomal drug delivery and, to a lesser extent, increased intracellular drug concentration. This increase in drug concentration was associated with better antitumor effect. This result strongly argues that increased liposome delivery will ultimately lead to greater intracellular drug accumulation and better antitumor effect.

We detected a significant positive correlation between doxorubicin concentration and radiotracer (expressed in %ID/g; Fig. 3). The overall thermal enhancement was slightly lower for Doxil in comparison with that of the radiolabeled tracer. The observed small differences might be less important in the clinic. Although the characteristics of the doxorubicin versus tracer uptake relationship may have to be further validated in humans, our work is proof of principle that the radiolabeled tracer can be used to estimate the effect of hyperthermia on liposomal drug uptake.

Future studies could be combined with an evaluation of tumor growth delay to investigate if the radiotracer could be used to predict treatment response. Because intratumoral drug levels are one of the best predictors of treatment response (19, 25), this method could give a better prediction for outcome than Doxil dose or plasma doxorubicin levels. Our approach may also obviate the need for a tumor biopsy to quantify the effect of hyperthermia on Doxil uptake. Additionally, a biopsy will only provide information on Doxil concentration in a small area of the tumor, whereas scintigraphy allows estimation of the hyperthermic effect across the entire tumor.

Scintigraphic images are a planar integral over the tumor volume, with some attenuation from deeper structures. Thus, scintigraphy has some limitations for determining spatial variation in drug accumulation. In a recent study, we have shown that the main determinant of antitumor effect of a thermally sensitive liposome formulation comes from total amount of drug delivered to the tumor, not the spatial variation in drug delivery.[7] We do not know if this effect would be similar for a nonthermally sensitive formulation. Nevertheless, our data suggest that a method that can quantitatively measure total drug uptake may be of value prognostically. It remains to be seen whether characterization of the spatial distribution of drug is an independent determinant of antitumor effect.

There are more sophisticated imaging methods that could be used in the future should spatial resolution or attenuation effects prove to be important. Other methods that have been reported to image liposomal drug delivery are single photon emission tomography, positron emission tomography, computed tomography, or magnetic resonance imaging (25, 28, 40, 41). Single photon emission tomography imaging can provide three-dimensional data (40) and could be done using the current radiotracer. Positron emission tomography imaging would also provide a three-dimensional distribution; however, this would require the development of liposomes labeled with a positron-emitting isotope. A disadvantage of positron emission tomography radionuclides is their relatively short half-life, which makes it harder to trace the *in vivo* behavior of a drug for longer time periods (40, 41). Magnetic resonance imaging has very good spatial resolution and we have used this method to measure drug concentration distributions with a thermally sensitive liposome formulation (42). However, magnetic resonance–imageable liposomes that can be used clinically are far from being ready for use and overall costs are considerably higher than using a gamma camera. Consequently, scintigraphy remains a valuable, low-cost, and easily applicable tool (4, 19, 25).

Most of the results reported here are in terms of means. We would like to point out, however, that results would remain essentially the same if medians are used instead, although the group sample size is relatively small. Statistical adjustments for baseline differences in subjects when comparing intratumoral extravasation from different experimental groups might be warranted through the use of a linear regression approach. This was not done for this study because of the small group sample size. Such adjustments, however, could be considered in a follow-up trial.

In conclusion, intratumoral extravasation of a small tracer dose of radiolabeled liposomes can be imaged under normothermic and hyperthermic conditions, and can be used to estimate the magnitude of the achieved thermal enhancement of drug delivery with Doxil liposomes and potentially other drug liposome combinations that are labeled in this fashion. The tracer approach is a promising and readily applicable technique to estimate drug delivery, as well as effects of interventions aimed to increase liposomal accumulation such as local hyperthermia.

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A Tracer Dose of Technetium-99m–Labeled Liposomes Can Estimate the Effect of Hyperthermia on Intratumoral Doxil Extravasation

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