Efficacy of Liposomes and Hyperthermia in a Human Tumor Xenograft Model: Importance of Triggered Drug Release

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ABSTRACT

The tumor drug concentrations, drug distributions, and therapeutic efficacies achieved by three fundamentally different liposomes, nonthermosensitive liposome (NTSL), traditional thermosensitive liposome (TTL5), and low temperature sensitive liposome (LTSL); free doxorubicin (DOX); and saline in combination with hyperthermia (HT) were directly compared in a human tumor xenograft model. NTSL is a nonthermosensitive liposome in the physiological temperature range, TTS is a traditional thermosensitive liposome that triggers in the range of −42–45°C and releases drug over ~30 min, and LTSL is a new low temperature sensitive liposome that triggers in the range of ~39–40°C and releases drug in a matter of seconds. Because of the different attributes of the liposomes, it was possible to delineate the relative importance of liposome drug encapsulation, HT cytotoxicity, HT-drug interaction, HT-induced liposomal delivery, and HT-triggered liposomal drug release in achieving antitumor activity. Athymic nude mice bearing the FaDu human tumor xenograft were given a single i.v. dose of 5 mg/kg of DOX (free drug or liposome encapsulated), and the tumors were then heated to either 34°C or 42°C for 1 h at 34°C. All treatment groups were similar, achieving low concentrations of DOX (0–4.5 ng/mg). At 42°C, the LTSL (25.6 ng/mg) achieved the highest DOX concentration (P < 0.04), but all three liposomal formulations (7.3–25.6 ng/mg) were higher than saline or DOX (0–0.7 ng/mg; P < 0.02). LTSL + HT was also the only group that resulted in significant amounts of DNA-bound DOX (nitrate silver-extractable fraction; P < 0.02). Tumor tissue sections were visualized for DOX fluorescence to investigate the local distribution of the drug in the tumor and confirm the relative drug concentrations based on fluorescence intensity. There was relatively little fluorescence seen with treatment groups at 34°C. At 42°C, the LTSL showed the most DOX fluorescence (P < 0.01), and the fluorescence, although not homogenous, was pervasive throughout the tumor sections. Therapeutic efficacy of treatments was determined from tumor growth time. At 34°C, the only treatment group significantly better than the saline group (9.8 days) was the NTSL group, with a growth time of 20.9 days (P < 0.02). At 42°C, all three liposomal formulations were more efficacious than DOX. LTSL + HT had the longest growth time (51.4 days) and the most number of local controls at 60 days (six of nine tumors). With HT, the DOX concentrations and fluorescence were tightly correlated with tumor growth delay, indicating that adequate (increased) drug delivery can be predictive of therapeutic effect. Overall, the LTSL + HT group showed the largest DOX concentration, the highest and most pervasive DOX fluorescence, and the most antitumor effect. Thus, HT-triggered liposomal drug release may account for the largest differential therapeutic effect and demonstrates the importance of rapid drug release from the drug carriers at the tumor site.

INTRODUCTION

One of the important factors in determining the effect of a therapeutic agent is achieving adequate local delivery to the tumor site. Because of the precarious blood supply and often high interstitial fluid pressure, many cancer chemotherapeutic agents are not effectively delivered to the tumor region (1). This is compounded by the need for almost 100% cell kill to effect a cure (2). The tumor vessel wall represents a significant barrier for many therapeutic agents (3), and nonspecific delivery can lead to significant systemic toxicities and a low therapeutic index that is often seen with current cancer chemotherapy that use injected free drug.

In the search to overcome these delivery issues, liposomes have been identified as promising carriers for therapeutic agents in the treatment of cancer (4, 5). By encapsulating cancer chemotherapeutic agents within liposomes, preferential delivery to the tumor can be attained (6). Initially, liposomes were plagued with rapid opsonization and uptake by the reticuloendothelial system, resulting in a short circulation time on the order of minutes. This has largely been resolved by incorporation of lipid-grafted PEG1 into the liposome membrane, which reduces opsonization and increases the circulation time to hours or days (7). PEG also increases vascular permeability to liposomes facilitating increased accumulation of drug containing liposomes in tumor tissue (8). Despite these advances, current clinically approved liposomal formulations (6) have still resulted in only modest increased efficacy for the treatment of cancer (9, 10). The actual advantage of current liposomal formulations is reduced toxicity and not increased therapeutic effect. Increasing the circulation time of liposomes has not been sufficient, and other modifications to liposomal therapy are needed to improve the treatment efficacy. Efforts to design liposomes that are pH sensitive, temperature sensitive, antibody targeted, or fusogenic have all been pursued with various degrees of success (11).

HT has been used to modify the local tumor environment to increase liposomal drug delivery to tumors (12). Although classically viewed as a form of adjuvant therapy to increase the efficacy of radiation and chemotherapy, HT can be applied to augment liposomal drug delivery by increasing tumor blood flow and microvascular permeability. At temperatures of 41–43°C, HT has been shown to increase blood flow (13) and oxygenation (14). HT has also been shown to increase permeability of tumor vessels to antibodies (15–18), ferritin (19), and Evans blue dye (20). More specifically, HT has been shown to increase permeability of tumor vessels to liposomes (21).

Additionally, HT can be used as a modality for increasing liposomal drug delivery to tumor by triggering release of drug from the liposome. Temperatures of 40–43°C have been shown to trigger drug release from specially designed thermosensitive liposomes (22–24), making it possible to release liposome contents at the heated site. Besides targeting and triggering release in liposomes, preclinical data have indicated that several cancer chemotherapeutic agents in combination with HT have supra-additive cytotoxic effects (25–29). Fur-

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3The abbreviations used are: PEG, polyethylene glycol; HT, hyperthermia; DOX, doxorubicin; NTSL, nonthermosensitive liposome; TTS, traditional thermosensitive liposome; LTSL, low temperature-sensitive liposome; MPDC, 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; HSPC, hydrogenated soy sn-glycero-3-phosphocholine; DSPE-PEG2000, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-PEG2000; HER, HT enhancement ratio.
thermore, HT itself has been shown to be directly cytotoxic (30). The therapeutic benefits from liposomes and HT individually, coupled with the potential advantages seen by their combination, make the use of the two modalities together an attractive method for drug delivery to tumors.

There have been many studies showing increased drug delivery to the tumor when the drug is encapsulated in a liposome and administered with HT (31–33). The therapeutic efficacy of combination HT and liposomal drug delivery has also been shown with several drugs, ranges of thermal doses, different tumor models, and various liposomal formulations (34–36). These positive findings are difficult to evaluate across studies because they have usually been limited to one liposome formulation. Thus, the different treatment regimens have not been directly compared in the same system, and the characteristics of an optimal liposome formulation remain poorly defined (12).

This report directly compares three long-circulating liposomal formulations with and without HT by tumor drug concentrations, tissue fluorescence, and growth delay using a human tumor xenograft model. The three different liposomes represent fundamentally different classes of liposomal carriers that can be used in combination with HT. The NTSL (21) is not thermosensitive; the TTSL (37) is thermosensitive, but only in the range ~42–45°C and releases drug over ~30 min; and the LTSL (38) is thermosensitive at the range of ~39–40°C and releases drug in a matter of seconds. Some of the results in this report have been published in a preliminary form (38), where the focus was to introduce the LTSL formulation, its physical properties, and effects on tumor growth delay when combined with HT. This report will provide detailed analysis of tumor drug concentration, tissue drug fluorescence, and growth delay results that have not been presented so that the key factors for optimal combination HT and liposomal therapy can be better understood. Using direct comparison of saline, DOX, NTSL, TTSL, and LTSL with and without HT, it was possible to determine the relative importance of liposomal drug encapsulation, HT cytotoxicity, HT-drug interaction, HT-induced liposomal delivery, and HT-triggered liposomal drug release to therapeutic efficacy.

MATERIALS AND METHODS

**Liposomes.** MPPC, DPPC, HSPC, distearoyl-sn-glycero-3-phosphatidylcholine, cholesterol, and DSPE-PEG-2000 were purchased from Avanti Polar Lipids (Alabaster, AL). DOX was purchased from Sigma Chemical Co. (St. Louis, MO). There were three liposomal DOX formulations prepared: NTSL, TTSL, and LTSL. The NTSL was composed of HSPC:cholesterol:DSPE-PEG-2000 in the molar ratio of 100:50:0:3 (21). The TTSL was composed of DPPC:HSPC:cholesterol:DSPE-PEG-2000 in the molar ratio of 100:50:30:6 (37). The LTSL was composed of DPPC:MPPC:DSPE-PEG-2000 in the molar ratio of 90:10:4 (37). All of the liposomes contained DOX and incorporated PEG in the membrane to achieve a long-circulation time. Lipid vesicles of required composition and size (~140 nm by dynamic light scattering) were prepared by the lipid film hydration and extrusion method (39). Encapsulation of DOX into the liposomes was carried out using the pH gradient-driven loading protocol (40).

**Mice and Tumors.** Homozygous NCr athymic nude mice (20 ± 3g) were purchased from Taconic (Germantown, NY). Animals were housed in appropriate isolated cages with sterile rodent food and acidified water ad libitum and a 12-h light/dark cycle. A human squamous cell carcinoma xenograft line, FaDu, was used in this study. The right lower leg of each mouse was inoculated s.c. with 1 × 10^6 single-cell suspension in a volume of 50 μl. Tumors were allowed to grow to 4–6 mm in diameter before starting treatment. Mice were carefully monitored for general well-being, weight, and tumor volume. Any mice exhibiting weight loss ≥15% of the initial weight were scheduled to be euthanized, but no animals fit this criterion. All protocols were approved by the Duke Institutional Animal Care and Use Committee.

**Treatment Protocol.** Mice were stratified by tumor volume and randomized to 1 of 10 treatment groups: saline, DOX, NTSL, TTSL, and LTSL; at 34°C or 42°C for 1 h. Except for the saline group, all treatment groups were given an equivalent single dose of 5 mg/kg of DOX. Mice were anesthetized with an i.p. injection of pentobarbital (80 mg/kg). This dose of anesthesia provided adequate immobilization for the 1-h heat treatment period, and no redosing was necessary. The mice were then given an injection of 100 μl of treatment solution via the tail vein. Immediately after injection, the mice were positioned in specially designed holders that allowed the isolated leg tumor to be placed in a water bath for 1 h. The isolated leg tumor was covered in a thin plastic sleeve to prevent excessive water absorption and subsequent limb edema (18). The water bath temperature was set to 35°C or 43°C, temperatures that had been previously calibrated to give tumor temperatures of either 34°C or 42°C, respectively, depending on the treatment group (18).

**Tumor Drug Concentrations.** Tumors were surgically excised (six tumors/group) immediately after the 1-h water bath treatment and frozen at −70°C. Samples were then evaluated via a liquid extraction of homogenates with chloroform and the addition of silver nitrate to ascertain total DOX concentrations (41). This method has been shown to extract >95% of DOX present in tissue. The presence of silver nitrate allows for the extraction of DOX bound to DNA and RNA that cannot be extracted with only chloroform. Samples were also extracted with only chloroform for comparison to determine the amount of DOX bound to DNA and RNA. Subsequently, concentrations of DOX in the tumor samples were quantified using a modification of a previously published gradient, reverse phase-high performance liquid chromatography assay with fluorescence detection (42). High and low concentrations of DOX in quality control samples were required to be within 15% of spiked concentrations for an assay run to be acceptable. Unless specified, data are reported as the amount of total DOX (extracted with chloroform and silver nitrate) in each sample per weight of extracted tumor tissue. The average value of six treated tumors is given for each of the 10 treatment groups.

**Tumor Fluorescence Sections.** Tumors were surgically excised (six tumors/group) immediately after the 1-h water bath treatment, and two 4-μm-thick frozen sections from each sample taken at different levels of the tumor were prepared. The tumor sections were examined under fluorescence microscopy (HS46; Zeiss) for DOX. Using an objective with ×20 and epi-illumination with light from a 100-W mercury lamp passing through a dichroic filter set suitable for DOX, images of the tumor sections were captured and analyzed off-line for fluorescence intensity. All tumor sections were analyzed with image processing software (NIH Scion Image) under the same settings and reported in arbitrary fluorescence intensity units.

**Tumor Growth Delay.** Mice were treated by 1 of the 10 treatments (8–12 mice/group): saline, DOX, NTSL, TTSL, and LTSL; at 34°C or 42°C for 1 h. Animals were weighed, and tumors were measured 3 times/week. Tumor volume was determined using the equation: volume = (width)² × length × π/6. Tumor measurements were taken by one individual and performed in duplicate to confirm measurements. The individual measuring the tumors was blinded to the treatment groups. Animals were followed until five times the initial tumor volume was reached or 60 days after treatment, at which point they were euthanized. Growth times are reported as the average of all animals in a treatment group.

**Statistics.** The Mann Whitney U test was used to determine statistical significance for all comparisons except for evaluating 60-day local control, where the Fisher exact probability test was used.

RESULTS

**Tumor Drug Concentrations.** The goal of these experiments was to determine the actual amount of DOX delivered to the tumor for each treatment after injection and 1 h of HT. Total DOX concentrations extracted with chloroform and silver nitrate include the bound and unbound fractions of the drug. Bound drug includes DOX bound to protein, membranes, DNA, or RNA. Unbound DOX concentrations reflect the amount of unbound drug in the tumor volume, either in the tumor interstitium or still entrapped in a liposome. Extraction with chloroform only does not determine the amount of DOX bound to DNA, RNA, and some proteins. Because DOX is ~80% bound in vivo across a wide range of concentrations (43), most DOX that is released from a liposome becomes bound. The total DOX tumor concentrations...
(concentration extracted with chloroform and silver nitrate) are reported unless otherwise stated.

At 34°C, the DOX concentrations achieved by all of the treatments were similar. The mean values ranged from 0 to 4.5 ng/mg, with saline being the lowest and the TTSL being the highest (Fig. 1). DOX concentrations at 42°C for three liposomes (NTSL, 8 ng/mg; TTSL, 7.2 ng/mg; and LTSL, 25.6 ng/mg) were higher than saline (0 ng/mg) and free DOX (1 ng/mg; \( P < 0.04 \)). In addition, the DOX concentration achieved by the LTSL at 42°C was significantly more than the other two liposomes (\( P < 0.04 \)).

All three liposomes achieved higher DOX concentrations at 42°C than at 34°C (\( P < 0.05 \)), likely because of increased liposomal delivery to the tumor. The LTSL showed the largest difference in DOX concentration between 42°C and 34°C, reflecting the significant liposomal drug release. Time points beyond 1 h were not taken because this study was mainly focused on the immediate effects of HT (given over 1 h) on liposome delivery and drug release.

DOX extraction from tumor tissue was done with and without silver nitrate (chloroform only; Fig. 1). In all groups except for LTSL + HT, there was no significant difference in DOX concentration with or without silver nitrate, although the presence of silver nitrate tended to increase the amount of DOX extracted in most of those groups. Tumors treated with LTSL + HT showed a significant difference in DOX concentration when extracted with silver nitrate (25.6 ng/mg) compared to without silver nitrate (13.1 ng/mg; \( P < 0.02 \)), indicating that there was a substantial amount of DOX bound to DNA and RNA in these tumors.

**Tumor Fluorescence Sections.** The goal of examining tumor tissue sections was to directly evaluate the relative levels of DOX and the local distribution of DOX achieved in the tumor immediately after treatment. All values are denoted in arbitrary DOX fluorescence intensity units, and the control group was used to determine background fluorescence. At 34°C, DOX tumor sections (0.89 units; Fig. 2B) showed slightly more fluorescence than the control group (Fig. 2A; \( P < 0.02 \)). All three liposomal formulations (Fig. 2, C–E) were essentially equivalent, ranging from 2.23 to 2.43 units, but were all more fluorescent than DOX (\( P < 0.01 \)). Overall, the treatment groups at 34°C were low in DOX fluorescence intensity. HT did not result in any difference in fluorescence for the control group. At 42°C, DOX tumor sections (0.99 units; Fig. 2G) were again slightly more fluorescent than the saline group (\( P < 0.03 \)). All three liposomal formulations (Fig. 2, H–J) were more fluorescent than DOX (\( P < 0.001 \)).

**Tumor Growth Delay.** The goal of this study was to compare growth times (time to reach five times initial tumor volume or 60 days after treatment) across liposome types, with and without HT (42°C for 1 h).

Within the normothermic group (34°C), the growth time for tumors

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**Fig. 1.** Tumor DOX concentrations of all treatment groups after i.v. injection of DOX (free and encapsulated) and 1 h of HT. Bar, SE.

**Fig. 2.** Representative tumor DOX fluorescence sections. Tumor sections were prepared immediately after treatment, and fluorescence intensities are noted in arbitrary units. Numbers in parentheses, SE. A, control at 34°C. B, DOX at 34°C. C, NTSL at 34°C. D, TTSL at 34°C. E, LTSL at 34°C. F, control at 42°C. G, DOX at 42°C. H, NTSL at 42°C. I, TTSL at 42°C. J, LTSL at 42°C.
treated with saline was 9.8 days (Fig. 3A). DOX and two of three of the liposomal formulations (TTSL and LTSL) had growth times that were not statistically different from the saline group (9.8–13.5 days; Fig. 3, B, D, and E). An exception was the NTSL group, which had a growth time of 20.9 days (P, 0.02, compared with saline). The NTSL group also had the largest variation in growth times as seen in Fig. 3C.

At 42°C, the growth times of the saline group (19.8 days) and DOX (23.7 days; Fig. 4, A and B) were not statistically different. All three liposomal formulations in combination with HT were significantly more effective than HT + DOX or HT alone (P < 0.05). NTSL + HT and TTSL + HT had growth times of 31.9 and 35.1 days, respectively (Fig. 4, C and D). TTSL + HT had local control at 60 days in 1 of 12 tumors treated. LTSL + HT showed the most growth delay with a growth time of 51.4 days (P < 0.002; Fig. 4E). This group had local controls at 60 days in six of nine tumors treated. In this study, within all formulations, HT was statistically more efficacious than no HT (P < 0.05). The HER, defined as the ratio of the growth time at 42°C to that at 34°C, ranged from 1.75 to 4.51, with the LTSL having the largest HER (Table 1). None of the mice in any of the treatment groups showed any obvious signs of morbidity or weight loss, indicating that the single-dose drug + HT was well tolerated.

**Correlation between Tumor Drug Concentrations, Tissue Fluorescence, and Growth Delay.** Tumor drug concentrations and tissue fluorescence were compared with tumor growth delay. At 34°C, no significant correlation was found between tumor drug concentrations or tissue fluorescence and growth delay. Relatively little drug was delivered to the tumor either by free drug administration or liposomal drug delivery, and the slightly extended growth delay seen for the NTSL did not correlate with higher tumor drug concentrations.

At 42°C, tumor DOX concentrations and tissue fluorescence were tightly correlated with tumor growth delay, with correlation coefficients >0.98 (Fig. 5). Comparison of results with a second, previously published tumor growth delay experiment (38), are shown (Fig. 5A). Both correlations were similar with slopes near unity, indicating a very direct relationship between DOX concentration, fluorescence, and growth delay. Thus, when substantial tumor DOX concentrations or tumor DOX fluorescence was achieved (e.g., at 42°C), they were predictive of tumor growth delay.

**DISCUSSION**

The combination of HT and liposomal drug delivery for cancer therapy has been used in many preclinical models (44–46). Most of the studies in the literature have largely focused on a combination of HT and one liposome type. Although many of these studies have shown increased efficacy with HT and liposomal drug delivery, it is difficult to compare across studies because of the many variables associated with each study (drug, tumor type, thermal dose, and liposome type). Additionally, within a particular study, it is often difficult to delineate which factors contributed to the overall efficacy of the treatment. This study was designed to isolate the different factors that theoretically contribute to the efficacy of combination HT and liposomal drug delivery. They include liposomal drug encapsulation (i.e., versus free drug administration), HT cytotoxicity, HT-drug interaction, HT-induced liposomal delivery, and HT-triggered liposomal drug release.

**Contribution of Liposomal Encapsulation.** Results from all three liposome types (NTSL, TTSL, and LTSL) at 34°C demonstrate the effect of liposomal drug encapsulation on tumor drug delivery, DOX...
[Doxorubicin] is the concentration of DOX extracted by chloroform and silver nitrate (ng of doxorubicin/mg of tissue). The numbers in parentheses are SE. Fluorescence of DOX is denoted in arbitrary fluorescence intensity units. Growth time was defined as days to reach endpoint at 42°C/34°C. Local control was defined as no tumor present at 60 days after treatment.

A

34°C (Fig. 3), the effect of HT cytotoxicity could be isolated. HT alone (42°C for 1 h) showed significant growth delay (10 days), and every treatment group in combination with HT was more efficacious than without HT (P < 0.05). These findings are consistent with the literature regarding HT cytotoxicity (30) and HT interactions with liposomes (12).

Contribution of HT-DOX Interaction for Free Drug Administration. There have been several studies examining the interaction of HT and chemotherapeutic agents (26). With some agents, supra-additive effects have been seen with HT (26, 27). For DOX, the current literature is not definitive. There have been studies showing HT + DOX synergy (25, 47, 48) and others showing only additive effects (49–51). Still others describe a threshold temperature of 42°C, above which synergy is seen (52). The tumor DOX concentrations and the DOX fluorescence for the DOX treatments at 42°C showed no activity at 34°C or 42°C compared with saline, it can be concluded that free DOX, given at this dose, showed no activity in this tumor model. Higher doses of DOX (7.5 and 10 mg/kg) were also tested in a pilot study but resulted in significant toxicity to the animals (data not shown). On the basis of these results, 5 mg/kg was determined to be the maximally tolerated dose.

Contribution of HT-induced Liposomal Delivery. To evaluate the contribution of HT-induced liposomal delivery, the NTSL + HT group was examined. HT might cause additional delivery of NTSL to the tumor because of increased tumor blood flow (13, 54), vascular tissue fluorescence, and therapeutic efficacy. Under normothermic conditions (34°C), there was little difference among liposomal treatment groups (tumor DOX concentrations, DOX fluorescence, or growth time) except for the NTSL group growth time (Table 1). Presumably all three liposome types, which are all long circulating and of similar size, would be equally likely to extravasate into the tumor interstitium because of the permeable tumor microvasculature. The DOX concentrations and fluorescence attained immediately after treatment by all three liposomes were similar, supporting this interpretation (Figs. 1 and 2). This degree of extravasation, however, for the contribution of HT-induced liposomal delivery, the NTSL at 42°C significantly better than all other treatment groups, P < 0.01. A portion of this table was modified from Needham et al. (38).

### Table 1 Comprehensive summary data for tumor DOX concentration, DOX fluorescence, and growth delay

<table>
<thead>
<tr>
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<th>T = 34°C</th>
<th>T = 42°C*</th>
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<tr>
<td></td>
<td>saline</td>
<td>DOX</td>
</tr>
<tr>
<td>[Doxorubicin]</td>
<td>0 (0)</td>
<td>1.48 (1.02)</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>0 (0.12)</td>
<td>0.89 (0.26)</td>
</tr>
<tr>
<td>Growth time</td>
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<td>13.5 (1.37)</td>
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<td>Growth delay</td>
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<td>HT growth delay</td>
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<td>10.2</td>
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<td>HER</td>
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<td>Local control</td>
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* Within treatment groups, HT (42°C) was always significantly better than no HT (34°C), P < 0.05.

* LTSL at 42°C significantly better than all other treatment groups, P < 0.01.

A portion of this table was modified from Needham et al. (38).
Fig. 6. Schematic of possible mechanisms involved in combination HT and liposomal therapy for solid tumors. A, liposomes (blue circles) preferentially extravasate from pores in tumor vessel walls. B, HT increases tumor pore size and thus increases tumor liposome extravasation. C, HT can trigger drug (yellow) release from liposomes in the tumor vessel. D, HT can trigger liposomal drug release in the tumor interstitium. E, HT can be directly cytotoxic to tumor cells.

permeability (17–20), or increased vascular pore size (55). Because the NTSL cannot be triggered to release drug, its therapeutic effect with HT is limited to HT cytotoxicity, HT-induced liposomal delivery, and any interaction between DOX that is released and HT. The NTSL does not release drug because of HT; therefore, it is unlikely that tumor cells were exposed to HT and significant levels of drug simultaneously. The NTSL tumor drug concentrations show approximately double the DOX tumor concentrations at 42°C (8 ng/mg) compared with that seen at 34°C (4.4 ng/mg; P < 0.05). This ratio is also seen for the tumor DOX fluorescence (42°C = 4.93 and 34°C = 2.38; P < 0.01). The results show a tumor growth delay of 11 days for NTSL + HT (Fig. 4C), but this is not significantly different from the effect attributable to HT alone (10 days). Therefore, although there may have been increased total liposome delivery with HT, this effect was not significant enough to translate into increased therapeutic efficacy for the NTSL because of the lack of significant release within a relevant time frame (i.e., DOX remained in the liposome). There have been several studies using NTSL showing some increased efficacy in combination with HT (36, 56). Despite these results, the effects of HT on tumor blood flow and vascular permeability, as discussed in the “Introduction,” are not always consistent. In different models, HT has not led to increased tumor blood flow (57) or vascular permeability (58, 59).

Contribution of HT-triggered Liposomal Drug Release. Using two temperature-sensitive liposomes (TTSLs and LTSLs) that have different triggering temperature ranges and release rates, we were able to explore the role of HT-triggered liposomal drug release in drug delivery and how differences in release characteristics affected efficacy. Triggered release occurs because of a lipid membrane phase transition that results in increased permeability across the liposome membrane (60). A peak in the membrane permeability occurs coincident with the midpoint of the gel-liquid crystalline transition (61). It is important to note that although the peak in permeability occurs at this midpoint, drug permeability can be appreciable 1°C or 2°C below this point. The TTSL starts drug release in the range of ~42–45°C, and 40% content release is achieved within 30 min at the appropriate temperature (38). This release temperature is not clinically optimized because most clinical HT treatments yield nonuniform temperatures with averages of ~40–41°C and ranges from 40 to 43°C (62). As discussed above, the LTSL is a novel liposome composition that incorporates lysolecithin into the gel-phase lipid membrane, which acts to slightly lower the phase transition temperature of the lipid mixture to ~39–40°C (24). This lowered transition temperature then brings down the temperature at which drug starts to be released into a temperature range that is clinically attainable in the tumor. The LTSL also has a higher total percentage of drug release and a faster rate of drug release than the TTSL (38). Forty-five % of content release occurs within 20 s, which may be expected to enable higher local concentrations of drug to accumulate in the tumor and even be released in the blood stream as the liposomes are circulating in the tumor. The results from this study confirm this expectation.

The highest DOX concentrations and fluorescence achieved were seen with the LTSL + HT group. It was the only group to show significant DOX binding to DNA and RNA (determined by the difference in the amount of DOX extracted with chloroform compared with that extracted with chloroform and silver nitrate; P < 0.02). DNA binding is one of the main mechanisms by which DOX exerts cytotoxicity (63). These differences between the LTSL and other liposomes reflect the large amount of DOX released by the LTSL at 42°C and may explain the significant therapeutic effect seen with this group. The large amount of DOX released by the LTSL most likely occurs by two mechanisms: tumor interstitial release and vascular release.

If LTSL extravasation and subsequent drug release were the only mechanisms for LTSL drug delivery, then the DOX concentration achieved should be similar to the TTSL and NTSL because all three of these liposomes have PEG and are similar in size. However, the DOX concentration achieved by the LTSL at 42°C was much higher than other groups, and an additional mechanism for LTSL drug delivery is likely present. This other mechanism is most likely LTSL release of drug in the tumor vasculature. A simple estimation of LTSL tumor circulation time can be useful for elucidating this mechanism of intravascular drug release. Erythrocyte velocity in tumor vessels has been shown to be approximately 0.1–0.2 mm/s (64). Assuming that LTSL velocity is similar to erythrocyte velocity in tumor vessels, in a 1-cm diameter tumor, LTSL will travel through the entire diameter of the tumor in 50–100 s. This time frame is long enough for LTSL to release its contents. Because most tumor vasculature is chaotic and
tortuous, LTSL circulation would most likely be even longer than estimated in this calculation. This occurrence of drug release in the local tumor blood stream would result in high intravascular drug concentration and the resultant concentration gradient that would drive drug into the tumor interstitium. The combination of interstitial and vascular drug release by the LTSL explains the high DOX concentration achieved. The TTSL is unable to release drug in the tumor vessels for two main reasons: time frame and vessel temperature. The estimated tumor circulation time for the TTSL would be similar to the LTSL but not long enough for appreciable TTSL drug release (<1800 s). Additionally, the TTSL would need to be continuously exposed to intravascular temperatures of 42°C. This condition is unlikely when the tumor interstitium is heated to 42°C (i.e., the vessel temperature would most likely be <42°C due to blood flow).

For both temperature-sensitive liposomes, augmented release of drug translated into an increased therapeutic effect (Table 1). The HT growth time for the TTSL was 24 days ($P < 0.02$). In the situation of the TTSL + HT group, heating to 42°C for 1 h, although suboptimal because of the higher transition temperature of the liposome, was sufficient to trigger some drug release (Figs. 2I and 4D). This drug release then translated into efficacy by increasing the growth time in addition to 60-day local control in 1 of 12 tumors. HT also triggered the LTSL, but to a larger degree. LTSL + HT led to a HT growth time of 40 days ($P < 0.01$) and 60-day local control in six of nine tumors (statistically different from NTSL and TTSL at 42°C, $P < 0.01$; Table 1). The higher efficacy for the LTSL compared with the TTSL is likely attributable to the significant tumor drug levels achieved in a short time frame, which is a function of the lower triggering temperature, the higher total percentage of drug release, and the faster rate of drug release. These characteristics allow for more elevated tissue drug concentrations (Fig. 1) in the tumor and help overcome any potential threshold dose for efficacy. Therefore, although DOX levels are increased with the NTSL and TTSL + HT, it is the difference of triggered and rapid drug release of the LTSL that appears to be instrumental in achieving sufficient concentrations of drug to allow for a larger therapeutic effect.

One of the many drawbacks to cancer chemotherapeutic regimens is the associated toxicity for normal tissues. In this situation, single-dose treatment of free or liposomal DOX (5 mg/kg) in combination with local HT (42°C for 1 h) was well tolerated. There were no cases of weight loss ≥15% of the initial weight and, in most cases, the animals gained weight during the study. In the case of the LTSL at 42°C, this further strengthens the case for a very high therapeutic index.

**Correlation between Tumor DOX Concentrations, DOX Fluorescence, and Growth Delay.** Because the groups with the highest and lowest tumor DOX concentrations and tumor DOX fluorescence yielded the largest and smallest growth delay, the possibility of a correlative relationship between tumor drug concentration, tumor DOX fluorescence, and growth delay became apparent. When correlating the relationships between these three results for the groups that received HT, the correlation coefficients were $>0.98$, and the slopes were all similar to unity (Fig. 5). These tight correlations show that the actual tumor drug concentration and/or tumor DOX fluorescence can be good predictors of each other and, ultimately, of therapeutic effect. These observations could become clinically useful as prognostic factors (e.g., determine tumor drug concentration or tissue fluorescence from a biopsy). In addition to these relationships, perhaps a threshold response needs to be overcome to obtain more long-lasting therapeutic effects. Only the LTSL at 42°C, which was triggered for significant drug release, resulted in consistent local controls (i.e., complete regressions at 60 days after treatment). No correlations could be made for the groups that did not receive HT because little tumor growth delay was seen.

In conclusion, this study aimed to explore whether liposome drug encapsulation, HT cytotoxicity, HT-drug interaction, HT-induced liposomal delivery, and HT-triggered liposomal drug release can contribute to more effective therapy. A schematic of these critical factors is presented in Fig. 6. Although much of the current literature has established that these factors are important, this study highlights the need for HT-triggered liposomal drug release at a high rate and at clinically attainable temperatures. It is also important to note that DOX alone was unable to delay tumor growth, but the same systemic dose of drug delivered in a liposome and triggered for release was able to produce a therapeutic effect. Although current liposomal formulations may emphasize one or two of these factors, it is necessary to optimize all of these factors to obtain the highest therapeutic intervention.

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**REFERENCES**

Efficacy of Liposomes and Hyperthermia in a Human Tumor Xenograft Model: Importance of Triggered Drug Release
