A New Temperature-sensitive Liposome for Use with Mild Hyperthermia:
Characterization and Testing in a Human Tumor Xenograft Model

David Needham, Gopal Anyarambhatla, Garheng Kong, and Mark W. Dewhirst

Abstract

The single biggest challenge now facing drug delivery (for liposomes and indeed other carriers) is to initiate and produce release of the encapsulated drug only at the diseased site and at controllable rates. Our efforts have focused on developing a new thermal-sensitive drug delivery system, specifically for the local control of solid tumors. We describe here a new lipid formulation containing doxorubicin that has been optimized for both mild hyperthermic temperatures (39°C to 40°C) that are readily achievable in the clinic and rapid release times of drug (tens of seconds). This new liposome, in combination with mild hyperthermia, was found to be significantly more effective than free drug or current liposome formulations at reducing tumor growth in a human squamous cell carcinoma xenograft line (FaDu), producing 11 of 11 complete regressions lasting up to 60 days posttreatment.

Introduction

It is now well recognized that when systemic chemotherapy is used in the treatment of solid tumors, it is almost impossible to achieve therapeutic levels of drug at the tumor site without damaging healthy organs and tissues (1). One solution to this problem is to encapsulate the drug in a biocompatible material that can be injected into the blood stream with the intention of delivering drug to a diseased site, (e.g., solid tumor tissue). Four key requirements of drug carrier design are essential to the overall function and performance of the carrier system. These requirements are “Retain, Evade, Target, and Release” (2). Over the last 30 years, lipid-based drug carrier systems have been developed that can retain drug, evade the body’s defenses, and so target (passively and specifically) the interstitial tissue of tumors (3, 4). Conventional liposomes have been clinically evaluated (5) and approved in a variety of diseases, and the “Stealth” formulation has been approved for clinical use in Kaposi’s sarcoma (4). Undoubtedly, drug does leak out of liposomes that have accumulated in tumor tissue, and there are situations where such slow release has provided therapeutic benefit and may be efficacious (4). However, the ability to control and produce a burst release would be extremely advantageous and may prove to be an essential step in providing efficacious levels of drug in the tumor. The single biggest challenge, therefore, now facing drug delivery (for liposomes and other carriers) is to initiate and produce release of the encapsulated drug only at the diseased site and at controllable rates. For this to happen, the carrier/drug relationship must be triggered to change from the stable or kinetic trap of required composition and size (— 140 nm) were prepared by the lipid film hydration and extrusion method (10). Encapsulation of DOX into the liposome was carried out using the pH gradient-driven loading protocol (11).

Temperature-induced Release of DOX from Liposomes in Vitro. The release of entrapped DOX from liposomes at various temperatures between 30°C and 45°C and time points between 0 and 3600 s was determined by measuring the amount of entrapped DOX that was released from a given sample of liposomes as a function of time at a given temperature. Each test temperature was attained by rapidly heating each sample from room temperature. In this way, the experiment represents a temperature jump and release of contents at each test temperature. A 20-μl aliquot of incubated sample was withdrawn, and after suitable dilution to 1 ml, the fluorescence intensity was measured on a fluorescence spectrophotometer (Shimadzu, RF-1501) as described by Maruyama et al. (12) with minor modifications. The relative percentage of fluorescence intensity after incubation at different temperatures was calculated by comparison with the total fluorescence intensity obtained after disrupting the liposomes by adding 0.3 M HCl-50% ethanol to the samples. To provide an environment somewhat closer to the preclinical situ-

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4 The abbreviations used are: DOX, doxorubicin; MPPC, 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphatidylcholine; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; HSPC, hydrogenated soy sn-glycero-3-phosphocholine; DSPE-PEG-2000, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-polyethylene glycol 2000; NTSL, nonthermosensitive liposome DOX; TTSL, traditional thermosensitive liposome DOX; LTSL, lysolecithin-containing thermosensitive liposome DOX.
ation of the mouse tumor than simple buffers, all of the in vitro drug release experiments and differential scanning calorimetry (DSC7; Perkin-Elmer, 2°C/min heating rate) were carried out in the presence of 50% bovine serum.

Mice and Tumors. Homozygous NCr athymic nude mice (20 ± 3 g) were purchased from Taconic (Germantown, NY). Animals were housed in appropriate isolated caging 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 implanted s.c. with 1 × 106 cells in 50 μl of PBS. 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. Mice with weight loss ≥15% of the initial weight or tumor volume ≥1500 mm3 were scheduled to be euthanized, but no mice in this study met that requirement for early euthanasia. The Duke Institutional Animal Care and Use Committee approved all protocols.

Treatment. In study 1, mice were stratified by tumor volume and randomized to 1 of 10 treatment groups (8–12 mice/group): saline, free DOX, NTSLs, TTSLs, and the LTSLs, at 34°C or 42°C. In study 2, mice were stratified by tumor volume and randomized to one of four treatment groups (10–11 mice/group): saline, NTSLs, TTSLs, and LTSLs, all at 42°C. In both studies, the stratification by volume assured that there was an equal volume distribution within groups, as confirmed by statistical analysis (data not shown). Except for the saline group, all treatment groups were given an equivalent single dose of 5 mg/kg of DOX. Mice in all treatment groups were anesthetized with an i.p. injection of pentobarbital (80 mg/kg); treatment was administered in a volume of 100 μl via tail vein injection. This dose of anesthesia provided adequate immobilization for the 1-h treatment period. No redosing was needed. We did not observe any attempts to withdraw the foot from the water bath (as one would expect from a pain response) or movement of the animals during the treatment period. 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. Depending on the treatment group, the water bath temperature was set at 35°C or 43°C. These water bath temperatures have been calibrated previously to give tumor temperatures of either 34°C or 42°C, respectively (13).

Evaluation. Animals were weighed, and tumors were measured three times/week. Tumor volume was determined with 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 posttreatment, at which point they were euthanized. The Mann-Whitney U test and Fisher’s exact tests were used to determine statistical significance.

Results and Discussion

Materials Characterization of Temperature-sensitive Liposomes. As shown in Fig. 1a, tested in bovine serum, the release of encapsulated DOX from the new LTSL was extremely fast upon heating the liposomes to 42°C compared with the pure DPPC liposome.

The LTSL released ~45% of its contents in the first 20 s of being exposed to the elevated temperature of 42°C, compared with only 20% over 1 h for pure DPPC. Also shown for comparison are the more traditional thermal-sensitive liposome (TTSL; Ref. 9), and a NTSL (8). As reported previously by Gaber et al. (8) and checked here independently, the TTSL took 30 min to release ~40% of its contents, and as expected, the NTSL did not release any drug upon heating to 42°C.

Moreover, as shown in Fig. 1b, the onset temperature for LTSL-triggered release was relatively narrow and occurred mainly between 39°C and 40°C. This release profile fits within the limits of even the worst cases for average temperatures usually encountered using regional heating for deep-seated tumors. For example, when treated with externally applied radiofrequency-phased arrays, temperatures for prostate and ovarian cancers are nonhomogeneous and range from a minimum of 39.3°C to a median of 40.4°C and 39.7–41.6°C, respectively (14, 15). Compared with DPPC alone, the presence of MPPC in the liposome bilayer lowered the drug release temperature by almost 2°C and significantly increased the amount released, thus maximizing the release parameters for clinical use. As shown in the inset to Fig. 1b, for the DPPC:MPPC mixture, the contents release started to become significant at a temperature 1.5 degrees below the main peak of the gel to liquid crystalline bilayer phase transition. This indicates that although not at a maximum (see below), the enhanced permeability was sufficient to allow drug to be rapidly released at these relatively lower temperatures. For the TTSL, the triggered release temperature was broader, in the range 41°C to 43°C, slightly lower than that reported by Gaber et al. (9), and nevertheless slightly higher than what is easily attainable clinically. Once again, the NTSL showed little release of drug.

The concept that underlies the enhanced release of encapsulated drug from the liposomes relies on two properties of the lipid bilayer: (a) an increased bilayer permeability at the gel-to-liquid crystalline phase transition temperature (Tm) compared with either the solid or liquid phases; and (b) the ability of a water-soluble lysolipid component to desorb from the bilayer as the first lipid begins to melt.
Previous experiments (16) and theory (17) have shown that passive bilayer permeability has a sharp maximum, coincident with the maximum in the transition enthalpy attributable to mismatches in molecular packing, especially at the interfacial boundary regions of gel and liquid domains. How might this permeability be enhanced? We hypothesized that if a molecule could be included in the gel-phase bilayer that was also fairly soluble in the aqueous phase, then as the lipid melted, the molecule would desorb and possibly enhance the boundary defect formation, and with it the passive permeability to entrapped drug and other material (18). We have shown previously that lysolipids readily desorb from liquid-phase lipid bilayers once the vesicle is washed with lysolipid-free buffer (19, 20). We therefore made a mixed bilayer of DPPC doped with the head group and acyl chain-matched lysolipid MPPC. The new concept then is that MPPC is kinetically trapped in the ideally mixed solid phase and, at the gel-liquid crystalline phase transition, leaves the bilayer upon melting and enhances the permeability compared with the pure DPPC bilayer transition. As shown in Fig. 1, the presence of only a few mol% of MPPC contained in the gel-phase DPPC bilayer of the LTLSs increased both the rate and amount of drug released and showed minimal release at body temperatures of 37°C, where the membrane permeability barrier was essentially maintained. These characteristics of attainable and narrow triggering temperature, rapid “burst” release of drug, and high cumulative drug release would appear to offer potential advantages that could lead to an increased therapeutic effect over and above traditional chemotherapy and current liposomal and other drug carrier systems.

**In Vivo Testing in a Human Tumor Xenograft Model.** To test this idea, we carried out two tumor growth delay studies in a human squamous cell carcinoma tumor xenograft model (FaDu) and compared saline controls to free DOX and each of three liposome DOX formulations (NTSL, TTSL, and LTSL) ± hyperthermia (Table 1).

DOX was chosen because of its clinical activity against many solid tumors (21) and the existence of established liposomal DOX formulations (22). Tumors were grown in the hind limb of athymic nude mice and heated by immersing the leg in a water bath. In the first study, we compared the effect of various treatments on tumor growth at normal skin temperature of 34°C and at a mild hyperthermic temperature of 42°C for 1 h. A second study was performed to confirm the results, where animals were randomly assigned to one of four treatment groups (saline, NTSL, TTSL, or LTSL), all heated to 42°C. The dose of DOX was 5 mg/kg for both studies.

In the first study, saline control and free DOX were compared at 34°C and 42°C. The 34°C control group had a growth time (time to reach five times the initial tumor volume) of 9.8 days. The growth time of free DOX was 13.5 days, which was not significantly different from the control. At 42°C, the growth delay for control was 10 days longer than at 34°C, demonstrating some hyperthermic cytotoxicity. However, free drug at 42°C was equivalent to heat alone. This lack of DOX activity could be attributed to either insensitivity of this tumor line to DOX or lack of delivery of sufficient drug. In a pilot toxicity study, we observed tumor regressions at doses of 7.5 and 10 mg/kg, but the drug was too toxic to use at those doses. Five mg/kg was chosen as the maximum tolerated dose for free drug. Therefore, the free drug is in fact toxic to the FaDu tumor, if high enough doses are reached, and we conclude that the lack of free drug activity at 5 mg/kg was attributable to lack of delivery and not lack of activity.

The TTSL and LTSL groups also showed essentially no activity against this tumor at 34°C, with growth times of 9.8 and 13.5 days. The growth time for NTSL, however, was ~20.9 days, a 10-day growth delay compared with the control group (P < 0.02). This result might be expected because NTSls have the highest cholesterol content, which has been reported previously to result in some drug loss over time in serum (9).

Of the heated groups in both studies, the growth delay results for the LTSL were the most striking. As shown in Fig. 2d, the LTSL formulation resulted in the most impressive antitumor effect.

All tumors (11 of 11) achieved a complete response, and none had any regrowth, remaining disease-free up to 60 days after treatment. In the first LTSL study at 42°C, there were eight of nine regressions and six of nine without tumor at 60 days. TTSL (Fig. 2c) produced one disease-free animal at 60 days and one extended growth delay, recapitulating the results of the first study (1 of 12 local controls at 60 days). The NTSL and TTSL groups had extended growth times compared with the heat alone (Fig. 2, b and c; and Table 1; P < 0.05). Of note, none of the mice in any of the treatment groups showed any obvious signs of morbidity. Most animals gained weight during the posttreatment period. None of the animals treated at 42°C lost weight in either experiment. In the first experiment, one or two animals in each group treated at 34°C lost some weight, while the rest gained weight. The maximum weight loss for those individuals that lost weight after treatment at 34°C ranged from 6% for free drug to 12% for the NTSL group. Therefore, the single dose of drug ± hyperthermia was well tolerated, perhaps even better than when these drugs were given under normothermic conditions.

The increased tumor growth times seen with the NTSL and TTSL formulations and hyperthermia demonstrate the importance of using heat to enhance liposome accumulation in tumor tissue for carriers.

<table>
<thead>
<tr>
<th>Study 1</th>
<th>( T = 34^\circ C )</th>
<th>( T = 42^\circ C )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days to reach end point&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.8 (0.77)</td>
<td>19.8 (1.75)</td>
</tr>
<tr>
<td>Regressions&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0 (10)</td>
<td>3 (11)</td>
</tr>
<tr>
<td>Local control&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0 (10)</td>
<td>0 (11)</td>
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<tr>
<td>Saline Free-DOX NTSL TTSL LTSL Saline Free-DOX NTSL TTSL LTSL</td>
<td></td>
<td></td>
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<tr>
<td>Days to reach end point&lt;sup&gt;e&lt;/sup&gt;</td>
<td>20.9 (3.23)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>23.7 (2.64)&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Regressions&lt;sup&gt;g&lt;/sup&gt;</td>
<td>2 (10)</td>
<td>6 (12)</td>
</tr>
<tr>
<td>Local control&lt;sup&gt;h&lt;/sup&gt;</td>
<td>0 (10)</td>
<td>0 (12)</td>
</tr>
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<sup>a</sup> Within treatment groups, hyperthermia (42°C) was always significantly better than no hyperthermia (34°C); \( P < 0.05 \).

<sup>b</sup> The end point was defined as days to reach five times initial tumor volume or 60 days. Numbers in parentheses, SE.

<sup>c</sup> Statistically significant compared with saline at that temperature; \( P < 0.05 \).

<sup>d</sup> LTSLs at 42°C are significantly better than all other treatment groups; \( P < 0.05 \).

<sup>e</sup> Regression is defined as a reduction in tumor volume for two consecutive measurements. Numbers in parentheses, number of animals.

<sup>f</sup> Local control is defined as no tumor present at 60 days after treatment.
Tumor volume (\( V_t \)) or 60 days. The differences in local control rates between LTSL and TTSL were highly significant in both experiments (\( P < 0.0001 \)). The nonlysophosphatidylcholine liposomes translate into the large difference in growth times of drug released from LTSLs at 42°C are superior to TTSLs. These effects became apparent. As is shown in Fig. 1, the rates and amounts of release and the rapid release of drug in achieving the best antitumor efficacy when they act synergistically. By formulating and delivering the two drugs in the LTSL system and using mild hyperthermia, both drug release properties to the type of drug delivered. For example, the release of anthracyclines may be best suited to a single protracted release because such drugs bind avidly to cells and act in a non-cell cycle-specific manner. However, other cell cycle-specific agents, such as camptothecins and Vinca alkaloids, may benefit from multiple short pulses over extended times as the long-circulating, temperature-sensitive liposomes continue to accumulate in the tumor at 34°C, it is not likely that any lysolipid released is causing any systemic toxicity.

The presence of lysolipid in the DPPC bilayers then leads to three key features of facilitated drug release by LTSLs as compared with the other temperature-sensitive lipid formulations, TTSLs, or pure DPPC liposomes: a lowered bilayer phase transition temperature (Fig. 1b), an increase in the rate, and an increase in the amount of drug release (Fig. 1a). All of these features contribute positively to the overall therapeutic success of this new drug formulation.

We did not test empty LTSLs, either alone or in combination with free drug, in these experiments. These are controls that should be considered for future studies. It is possible that the lysolipid in the LTSLs could cause some cytotoxicity, which could add to the overall antitumor effect. This will be determined from ongoing studies evaluating the antitumor effect as a function of drug release in vivo. Because no weight loss was seen in the LTSL group treated at 42°C and the degree of weight loss is less than a nonlysolipid-containing liposome at 34°C, it is not likely that any lysolipid released is causing any systemic toxicity.

An important issue with relation to this type of application is to keep the drug in the tumor tissue and not allow it to be reabsorbed back into the microcirculation. The rapid tissue binding properties of DOX (24) make it an attractive drug in this respect. Other drugs, with less avid tissue binding characteristics, may be less effective. However, the overall effectiveness of this approach may also be dependent upon the mechanism of the antitumor effect. Given the characteristics of drug transport from microvasculature, the highest tissue concentration is likely to be perivascular. Thus, it is possible that direct vascular damage might be part of the antitumor mechanism. This being the case, then one would expect that this approach might be more effective than simply administering drug intratumorally, for example. Additional studies are under way to investigate this issue further.

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The high tumor cure rate achieved in this model makes this system very attractive to apply to other water-soluble and remote-loadable drugs and a variety of oncological as well as nonmalignant situations. Because of the very rapid release kinetics associated with this new triggered-release system, one potential use could be to match drug-release properties to the type of drug delivered. For example, the action of anthracyclines may be best suited to a single protracted release because such drugs bind avidly to cells and act in a non-cell cycle-specific manner. However, other cell cycle-specific agents, such as camptothecins and Vinca alkaloids, may benefit from multiple short pulses over extended times as the long-circulating, temperature-sensitive liposomes continue to accumulate in the tumor tissue. Another potential application could be for drugs that show enhanced efficacy when they act synergistically. By formulating and delivering the two drugs in the LTSL system and using mild hyperthermia, both drugs could be made to accumulate and release in the tumor at the same time. Nonmalignant diseases, such as psoriasis and rheumatoid arthritis, and the deployment of other agents, such as anesthetics, might also benefit from a temperature-triggered, local, and rapid release of drug.

that do not necessarily release drug rapidly and confirms previous studies (7, 8). Nevertheless, the importance of increased liposomal delivery and the rapid release of drug in achieving the best antitumor effect became apparent. As is shown in Fig. 1, the rates and amounts of drug released from LTSLs at 42°C are superior to TTSLs. These properties translate into the large difference in growth times in vivo between LTSLs (Fig. 2d) and TTSLs (Fig. 2c) at 42°C, thus showing the importance of enhanced drug release in achieving the best antitumor effect. Additional studies testing how this enhanced release in vivo is responsible for the improved antitumor effects of this formulation are in progress. If enhanced release is the primary mechanism underlying the improvement in effect, one could argue that it might be analogous to drug applications involving intra-arterial administration. The problem with this approach is that not all of the vascular supply to tumors is arteriolar (23). Therefore, one might not expect to achieve complete vascular coverage of the tumor with an intra-arterial approach. The liposomal approach is more likely to reach all vessels of a tumor, whether arteriolar or venular in nature, because the liposomes continue to circulate for several hours after administration.

An important issue with relation to this type of application is to keep the drug in the tumor tissue and not allow it to be reabsorbed back into the microcirculation. The rapid tissue binding properties of DOX (24) make it an attractive drug in this respect. Other drugs, with less avid tissue binding characteristics, may be less effective. However, the overall effectiveness of this approach may also be dependent upon the mechanism of the antitumor effect. Given the characteristics of drug transport from microvasculature, the highest tissue concentration is likely to be perivascular. Thus, it is possible that direct vascular damage might be part of the antitumor mechanism. This being the case, then one would expect that this approach might be more effective than simply administering drug intratumorally, for example. Additional studies are under way to investigate this issue further.

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Fig. 2. Individual tumor volume growth curves from the second study heating at 42°C for 1 h. Each line is an individual mouse in the treatment group. a, saline; b, NTSL; c, TTSL; d, LTSL. The growth delay was ended when tumors reached five times initial tumor volume (\( V_i \)) or 60 days. The differences in local control rates between LTSL and TTSL were highly significant in both experiments (\( P < 0.01 \)) or when the two studies were combined (\( P < 0.0001 \)).
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References

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