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RESEARCH ARTICLE

Preliminary Results of Combining Low Frequency Low Intensity Ultrasound and Liposomal Drug Delivery to Treat Tumors in Rats

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Ultrasound is a convenient trigger for site-specific drug delivery in cancer therapy. Nano-sized liposomes formulated from soy phosphatidyl choline, cholesterol, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000] and alpha-tocopherol were loaded with Doxorubicin (Dox) using a pH gradient. The liposomal suspension was infused through the tail vein of BDIX rats possessing bilateral intradermal DHD/K12 tumors on their hind legs. Then 20-kHz ultrasound was applied to only one of the tumors for 15 minutes. This therapy was repeated weekly for 4 weeks. The results showed that in five of six rats, the tumors regressed to non-measurable size within 4 weeks. A paired comparison of the normalized size of the insonated and non-insonated tumors in the same rat indicated that the insonated tumors were smaller ($p < 0.0001$, $n = 6$ rats, 21 pairs). This observation has significant potential for non-invasive site-specific therapy of solid tumors.

Keywords: Ultrasound, Drug Delivery, Liposomes, Doxorubicin, BDIX Rats, Rumor Model.

1. INTRODUCTION

Our research group has previously developed ultrasonically-activated micellar drug carriers that can control the delivery of Doxorubicin (Dox) in space and time by focusing ultrasound (US) non-invasively on the specific tissue to be treated.[1–3] For example, micelles consisting of Pluronic P105 (NanoDeliv®) stabilized using an interpenetrating network of *N*,*N*-diethylacrylamide (NNDEA)[1,2] can be easily loaded with Dox, and upon insonation, will release part of their payload *in vitro*.[3] Using an *in vivo* rat model, we have shown that encapsulated Dox efficacy against tumors can be enhanced in one region by weekly insonation for 15 minutes, while little or no effect of the chemotherapeutic drug is observed in an adjacent region.[4] Specifically, the growth rate of the ultrasonically-targeted tumor in a leg was reduced while a non-targeted (control) tumor in the other leg was less affected. Most importantly, the heart appeared to be spared from the known cardiotoxicity of Dox. More recently, we have reported that the encapsulated anti-neoplastic agent's activity is independent of acoustic frequency as long as the combination of frequency and power density produces a similar intensity of inertial cavitation.[5]

In this paper we report even better *in vivo* results when using a liposomal drug carrier in combination with low frequency ultrasound. A liposome is a vesicle composed of a surfactant bilayer enveloping an aqueous interior. Liposomes can carry hydrophilic drugs in their aqueous interior and hydrophobic drugs within their bilayer.[6,7] Liposomes containing poly(ethylene oxide) (PEO) chains extending from their surface are known as "stealth" liposomes.[10,11] The PEO chains prevent the opsonization of these carriers and allow them to circulate longer in the blood. Liposomes are structurally stable and can retain their integrity when introduced into the human body. There are many advantages of drug delivery from properly formulated stealth liposomes. (1) They are structurally stable and will not rupture when subject to physiologically relevant shear stresses. (2) They have a long shelf life. (3) They are stable in biological fluids (blood). (4) Their size can by tailored to meet the specific requirements. (5) Their surfaces can be decorated with targeting groups for improved efficacy of drug delivery.

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On the other hand, there are two limitations that a liposomal drug delivery system presents. First, only hydrophilic agents can be encapsulated inside their aqueous core; hydrophobic drugs that might partition to their lipid bilayer might disrupt their stability. Second, the drug eventually needs to escape from a liposome that has been designed to be fairly stable. There are some commercial nano-sized liposomes that carry Dox, and these systems rely on the passive breakdown of the liposome over time or the slow diffusion of Dox through the lipid bilayer. Thus a triggered release mechanism might be very useful for liposomal drug delivery.

Previous research has shown that US can release Dox from liposomes.[12–22] Modeling of the release profiles suggests that there might be 2 pathways of escape; one pathway is via gross liposome destruction or disassembly,[22, 23] and the other pathway is via ultrasonically-enhanced membrane permeability of intact liposomes.[18,20, 22, 24, 25] For example, Schroeder et al. propose that ultrasonic permeabilization of liposomes occurs via transient formation of pores in the membrane due to gas nucleation and expansion in the lipid bilayer, and that upon cessation of insonation the bilayer membrane heals.[22, 24, 25] Lin et al. were less bold in speculating a mechanism for membrane leakage, but suggested that ultrasound-enhanced leakage "involves the formation of membrane defects that act as sites of enhanced permeation."[8] Although the details of US-activated release from liposomes are still not fully elucidated, US increases the rate of drug release from liposomes *in vitro*.

In vivo research has also examined the benefit of combining US and liposomal drug delivery. For example, Frenkel et al. applied high intensity US of short duration to try to enhance the therapeutic effect of liposomal Dox in mouse models of cancer.[26–28] They applied medium frequency (0.5 to 1.5 MHz), high intensity US of short duration (seconds) to the tumors after systemic administration of the liposomal Dox. Their results in general showed some benefit over non-insonated controls, particularly when using temperature-sensitive liposomes.[9]

In the present research we employed a different modality of US than did Frenkel et al. We used low frequency, low intensity ultrasound of long duration (minutes) to treat tumors in a rat model. The drug-containing liposomal carrier presented in this paper was injected systemically into the blood stream, and ultrasound was applied to only one of two bilateral tumors. The initial therapeutic results surpass those reported previously.

2. MATERIALS AND METHODS

2.1. Materials

Soy phosphatidyl choline (PC, 20 mg/mL in chloroform), cholesterol (10 mg/mL in chloroform), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000] (DSPE-PEG2000, 10 mg/mL in chloroform) were purchased from Avanti Polar Lipids (Alabaster, AL). Sucrose, ascorbic acid, alpha-tocopherol and HEPES were purchased from Sigma-Aldrich (Saint Louis, MO). Doxorubicin hydrochloride was obtained as a powder (1:5 in Dox:lactose mass ratio) from Bedford Laboratories (Bedford, OH).

2.2. Dox in Stealth Liposomes

PC:cholesterol:DSPE-PEG2000:alpha-tocopherol at a mass ratio of 3:1:1:0.004 were dissolved in chloroform and placed in a 50 mL flask. They were dried by N_2 flow and vacuum drying in the flask. Four mL of ammonium sulfate solution (0.11 M) were added to the flask, which was subsequently heated in a 60 °C water bath. The flask was then sonicated (70 kHz, 2 W/cm^2) in a Sonicor 100 (Sonicor, Copiaque, NY) cleaning bath for 15 to 30 minutes until no lipid residue was left on the flask. This solution was sheared several times in a "gas-tight-syringe" (Hamilton Co., Reno, NV) with a 0.8 μm filter (Cole Parmer, Vernon Hills, IL). Finally, it was sheared twice using a 0.2 μm filter (Cole Parmer, Vernon Hills, IL).

A PD-10 Sephadex Spin Column was equilibrated with a sucrose/ascorbic acid/HEPES/NaOH solution (0.26 M sucrose, 5 mM ascorbic acid, 15 mM HEPES, pH = 7.5). Two mL of the liposome suspension were layered on each of two spin columns, the column spun at 1,000 rpm for 2 min, and the eluent was collected.

The liposomal suspension was diluted in sucrose/ascorbic acid/HEPES/NaOH buffer, and the size distribution was measured by dynamic light scattering at a scatter angle of 90 degrees using a *Brookhaven 90Plus* Particle Sizer (Brookhaven Instruments Co., Holtsville, New York). Results showed that the liposomes have an average diameter of 142 nm. One peak was observed at 90 nm and another smaller peak at 220 nm.

Powder equivalent to 20 mg Dox was dissolved in the sucrose/ascorbic acid/HEPES/NaOH buffer to a final concentration of 10 mg/mL. This solution was mixed with the spin column eluent (the liposomes) and incubated at room temperature for 24 hours. During this time the neutral Dox diffused into the liposomes, was protonated by the ammonium sulfate inside the liposome (pH∼4.5), and then condensed into a sulfate salt that could not escape the liposome.[10] Finally the external Dox was removed by passing the liposome suspension through an ion exchange column (Dowex) that trapped the free Dox and passed the liposomes. The solution was stored at 4 °C until use.

The final Dox concentration of the liposomal suspension was determined by lysing an aliquot of liposomes with isopropanol/HCl and measuring the absorbance in a spectrophotometer at 495 nm.

2.3. Cells

DHD/K12/TRb cells (European Collection of Cell Cultures Ref# 90062901, Salisbury, Wiltshire, UK) were grown at 37 °C, 5% CO_2, in RPMI supplemented with nystatin, gentamicin, 2 mM *l*-glutamine, and 20% fetal bovine serum. Cells were split 1:3 using 0.25% trypsin/EDTA when they reached confluency (about 3 days). DHD/K12/TRb is a metastatic colorectal tumor cell line originating from a 1,2-dimethylhydrazine-induced colon adenocarcinoma in BDIX rats, and has been shown to spontaneously generate tumors at the injection site.[11, 12]

2.4. Rat Model

Six four-week-old BDIX rats were inoculated intradermally on each hind leg with 25 μl of DHD/K12 cells at a concentration of 2×10^6 cells/ml. Infusion of the liposome-encapsulated Dox was administered when their tumors were at least 1 mm in diameter, usually after about 3 weeks of growth. Dox in stealth liposomes was injected intravenously (4.4 mg/kg) via tail vein. Ultrasound at 20 kHz was applied for 15 minutes and 1 W/cm^2 (temporal average) to only one tumor, while the other tumor acted as an untreated control. This treatment was repeated weekly for 4 weeks. Insonation commenced within 5 minutes of completion of infusion. Both tumor sizes were quantified every week following 15 minutes of insonation where the major and minor lengths of each tumor (insonated and control) were measured (a and b, with $a \geq b$) using calipers. Tumor volume (TV) was estimated by $TV = ab^2/2$.[12] The tumor volume data were fitted to an exponential growth model: $TV = A_0 e^{kt}$, where A_o is the volume on the first treatment day, and k is the growth rate constant. Because each tumor was a slightly different size on the first day, the TV values were subsequently normalized by dividing all values for a given rat by its tumor volume on the first day, TV_o. For more details on rat treatments, please refer to our previous publications.[4, 5]

Ultrasound was applied at 20 kHz with a Sonics and Materials VCX 400 (Newton, CT) employing a titanium probe with 3 mm diameter. A volume of ultrasonic gel was placed on the depilated skin over the tumor with the gel extending to the edges of the tumor and about 5 mm high. The probe tip was positioned within the gel exactly above the center of the tumor with the tip 2 to 2.5 mm from the skin.

The ultrasonic probe had been calibrated previously *in vitro* using a Bruel & Kjaer hydrophone (model 8103, Norcross, GA). In this calibration, the hydrophone was placed 3 mm below the probe tip in an 80-liter tank lined with absorbing rubber. The acoustic intensity received by the hydrophone was correlated with the setting on the instrument. For these experiments, the setting on the instrument was set to deliver 1 W/cm^2 at a distance of 3 mm in water.

3. RESULTS AND DISCUSSION

This study employed Dox sequestered in the "stealth" liposomes described above. Liposomal Dox was injected via tail vein, and the tumor was insonated continuously for 15 minutes at 1 W/cm^2 and 20 kHz. The tumor volume was estimated following the weekly injections and insonation treatments. Figure 1 shows the data from all of the rats in these experiments. The squares represent the normalized volumes of tumors receiving US, while the diamonds represent the normalized volumes of control tumors without insonation. In contrast to the data from rats receiving micellar Dox, these data all show that the tumor receiving US and liposomal Dox quickly regressed in size, usually much more than the control side. Five of six tumors regressed to non-measurable size within 4 weeks, and some within 2 weeks. An exponential growth model was applied to fit individual tumor growth data. Figure 2 shows a box plot comparing the growth rate constant (k) of non-insonated and insonated tumors. The box plot shows that there are obvious differences in growth rate.

We also performed a "global" regression of all of the data from the 6 rats to estimate a tumor growth rate constant for the exponential growth model. For the insonated and control tumors, the growth rate constants were −0.13/day and 0.012/day, respectively. These values indicate that there was some slow growth when liposomal Dox was administered without ultrasound, but there was significant regression when US was applied.

Our data set also allowed us to do a direct comparison of the sizes of the treated and control tumors in each rat each week. A paired comparison of the normalized tumor size in the same rat indicated that the insonated tumors were smaller ($p < 0.0001$, $n = 6$ rats, 21 pairs).

This study is not the first to investigate the combination of stealth ultrasound with liposomal Dox, but our procedures and results are very different than in previous studies. Frenkel and Dromi et al.[26, 28] used Doxil® and 1.0 MHz ultrasound to treat JC tumors in a mouse model. Although they reported that US enhanced the extravasation of the liposomes, their data showed that any enhancement in tumor treatment (reduced growth) was not significantly greater than Doxil® without US. Yuh et al.,[13] using a mouse model but with a different tumor (SCC7), reported that US slightly enhanced the effectiveness of Doxil® in reducing tumor growth. So what is the difference between these previous reports and our work reported above? The time, intensity and frequency were different in these experiments.

As far as the time of exposure, Frenkel et al.[14] applied very short pulses of US, and though they were applied over several minutes, the total exposure time (the "on" time) was only 10.8 seconds. Likewise, Dromi et al.[9] used short pulses adding up to only 12 seconds of exposure, while Yuh et al. had a total exposure time of 24 seconds.

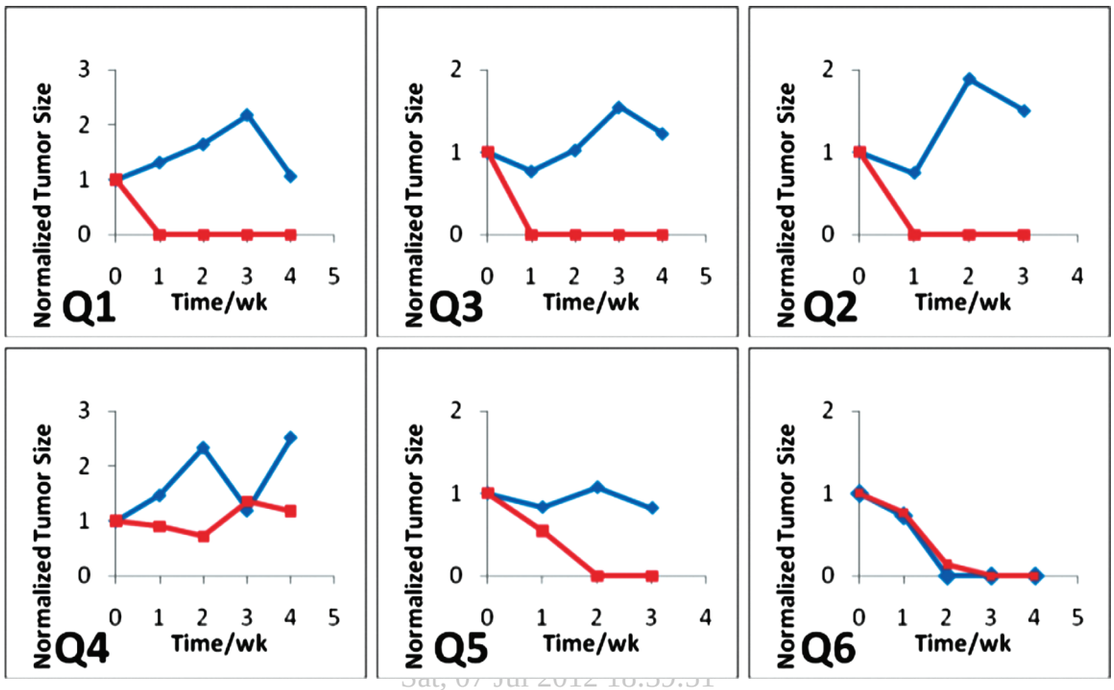

Fig. 1. Normalized tumor volumes of BDIX rats receiving liposome-encapsulated Dox (2.67 mg/kg) and 1 W/cm^2 US on one tumor only. The tumor size is normalized by dividing by the tumor size on the first day of treatment. The squares represent the tumor receiving US, while the diamonds represents the control tumor without insonation.

In our experiments with liposomal Dox, the ultrasound was applied continuously (no pulsing) for 15 minutes. This exposure is more than an order of magnitude longer than what has been employed in Frenkel, Dromi and Yuh's experiments.

The acoustic intensity applied in our experiments differs from what these groups used previously, since the long continuous exposures of our work require low intensities so that the tissue is not damaged. The intensity in this work was only 1 W/cm^2, compared to 124 W/cm^2 for Frenkel,[14] 1,300 W/cm^2 for Dromi,[9] and 1,114 W/cm^2 for Yuh,[13] which are 2 and 3 orders of magnitude higher than what we employed. Thus our insonation is mild and continuous, while theirs is pulsed, intense and short. Additionally, our results presented above were produced using 20-kHz ultrasound, while Frenkel and Dromi employed 1 MHz and Yuh used 1.5 MHz. These are 50 to 75 times higher in frequency. While this is a notable difference, we are hesitant to propose that frequency is the primary differentiating factor. Our previous work with Dox-containing micelles showed that reduced growth was observed at a wide range of frequencies, 20, 70 and 500 kHz, suggesting that, at least with micelles, frequency is a parameter of lesser importance.[5] While it might be possible that when using liposomes the difference between our positive results in tumor regression and the less positive results of others is due solely to frequency, we do not have sufficient data at present to make such a claim.

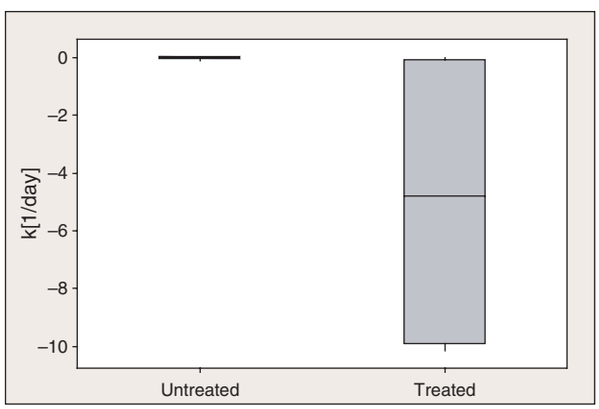

Fig. 2. Box plot comparing the growth rate constant, k, for treated versus untreated tumor sides. In the box plots, the mid-line represents the median value, while the upper and lower box boundaries indicate the bounds of the 1st and 3rd quartiles. The whiskers indicate the total range of data.

Because drug delivery via insonation usually involves cavitating microbubbles, one should examine the mechanical index employed in these experiments.[15] The mechanical index (MI) is defined as the peak negative pressure of the acoustic wave (in MPa units) divided by the square root of the center frequency (in MHz). The MI was 1.22 in our experiments, compared to estimates of 1.93, 6.24 and 4.72 in the experiments of Frenkel, Dromi and Yuh, respectively. However, we speculate that MI is less of a factor in our successful experiments than was the longer exposure time. Although the animal models are also different,

and the liposomes might be slightly different, we speculate that the longer duration of low intensity US is the primary difference responsible for the tremendous difference in therapeutic result. Further work is ongoing to validate this hypothesis.

This therapeutic result using Dox-containing liposomes is also much more effective than using Dox-containing micelles in the same rat tumor model. Similar experiments employing Dox-loaded micelles (at 2.67 mg/kg) produced a slight but statistically significant reduction in tumor growth rate at 476 kHz, from 0.038 ± 0.007 per day to 0.032 ± 0.013 per day.[5] The therapeutic results with micelles were much less pronounced than the results in which stealth liposomes were employed.

To summarize, therapy in a rat tumor model is very dependent upon the application of ultrasound and the type of carrier by which the Dox is delivered. Ultrasound combined with Dox delivered in our stealth liposomes is much more effective in reducing tumor growth than Dox delivered from our micelles. Low frequency, low intensity, and longer ultrasonic exposure appear to create a better therapeutic outcome than other approaches. More research is being performed to reveal the mechanisms by which ultrasound and liposomes effectively produce tumor regression.

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