

# Ultrasound drug targeting to tumors with thermosensitive liposomes

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**Abstract**— Induction of local tissue hyperthermia is emerging as a valuable tool in cancer therapy, as temperatures between 39–43°C are sufficient to trigger release of drug from thermosensitive liposomes (TSL), but is not harmful to normal tissue. Despite significant advances in spatial and dynamic control of ultrasound, temperature profiles in heated tissues are never homogenous, and an ideal TSL should achieve complete local release over the entire hyperthermia range. We have developed a TSL exhibiting a sensitive temperature release profile (39–43°C) with excellent stability at 37°C. We prepared a TSL composed of DPPC lipid and Brij78 surfactant, and loaded this hyperthermia-activated-cytotoxic (HaT) TSL with doxorubicin (DOX). EMT-6 breast tumors located on a Balb/c mouse footpad were instantaneously heated to 42–43°C using a 3.9 MHz planar transducer: body temperature did not elevate above 37°C, and complete remission of the EMT-6 breast cancer tumors was observed. Mice treated with standard DOX chemotherapy (at same 10 mg/kg dose as HaT) did not exhibit any tumor inhibition effects compared to control mice. By histological examination, no physiological damage to normal tissues was induced by ultrasound heating, and mice treated with HaT DOX regained normal tissue appearance and function post-treatment. This study confirms the benefit of coupling ultrasound induced hyperthermia with a sensitive TSL formulation.

**Index Terms**—ultrasound, hyperthermia, thermosensitive liposome, cancer

## I. INTRODUCTION

THE treatment of solid tumors with thermosensitive liposomes (TSL) has emerged as a viable clinical alternative to traditional chemotherapy, increasing the anti-tumor efficacy of existing chemotherapeutics, and concomitantly reducing side effects [1–7]. Drug-loaded TSL are administered intravenously to the bloodstream, and as the liposomes circulate within a locally heated tissue, a phase transition in the lipid membrane results in a burst-release of drug. The elevated local concentration of drug leads to

increased diffusion into tumor tissues, with reduced systemic exposure. Two clinical forms of heating are approved for use in cancer treatment: thermal ablation [8] and mild hyperthermia [9]. While thermal ablation can be applied to the debulking of large tumors, mild hyperthermia is better suited where tumors are located adjacent to sensitive tissues (such as arteries), or to tumors that are diffuse (e.g: recurrent cancer of the chest wall, pancreatic cancer).

Several TSL design challenges must be met to achieve effective drug release with mild hyperthermia: (1) homogenous heating of tumor tissue is difficult due to tissue heterogeneity and perfusion effects, and while the target temperature may be 43°C, tissues within the field of treatment may be cooler [10–11]; (2) as the approved temperatures (39–43°C) are mild, the TSL must begin to release just above physiological temperature 37°C, but remain stable in the non-tumor bloodstream; (3) blood residency time in tumors is short, so a TSL suited to hyperthermia temperatures must release drug within seconds. The most clinically advanced TSL is ThermoDox (Celsion, Phase II and III), composed of DPPC, lyso-PC, and DSPE-PEG<sub>2000</sub> (86:10:4, molar ratio): this three-part liposome encapsulates doxorubicin (DOX), a potent anticancer therapeutic [1–7], and is proceeding well through clinical trials for liver cancer and recurrent breast cancer of the chest wall ([www.celsion.com](http://www.celsion.com)). ThermoDOX treatment is currently coupled with radiofrequency (RF) ablation for liver, or focused microwave for breast cancer of the chest wall. However, the LTSL (lysolipid-TSL) only initiates rapid release at >40°C and is relatively leaky at 37°C [12], reducing the dose reaching the tumor, and increasing toxicity. Furthermore, RF techniques require insertion of a probe, and electromagnetic (microwave) heating is limited to superficial heating [13–15]. In contrast, tissue attenuation of ultrasound is moderate [13]; ultrasound can be highly localized with good depth penetration up to 10 cm [13]; ultrasound can be coupled with MR imaging technology to precisely localize treatment and follow response [16–17]. To advance TSL therapy, we have focused on developing a more temperature sensitive TSL to enhance drug release throughout the tumor, and coupled our novel TSL with an ultrasound heating modality suitable for screening a large number of animal models.

The novel TSL is composed of DPPC lipid (1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine) and Brij78 surfactant (96/4 mol/mol). Brij78 was selected to replace the lyso-lipid and

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DSPE-PEG<sub>2000</sub> components in LTSL, as its chemical structure contains functional moieties representative of these components. Brij78 is a polyethoxylated stearyl ether (similar in function to lyso-PC), and the PEG-component of this molecule protects the liposome from rapid clearance by the reticuloendothelial system (RES), extending its blood circulation time. Furthermore, the DPPC and Brij78 liposome composition undergoes thermal transition at 40.5°C, and accordingly, exhibits rapid release kinetics in the clinical hyperthermia range, but retains excellent stability at 37°C. This formulation (HaT: **H**yperthermia-**a**ctivated cyto**T**oxic) exhibits a more sensitive release profile at lower temperatures, and as a result, treatment with HaT improved DOX uptake in the heated tumor by 1.4-fold compared to LTSL, resulting in enhanced tumor regression after one single treatment at 3 mg DOX/kg [18].

## II. METHODS

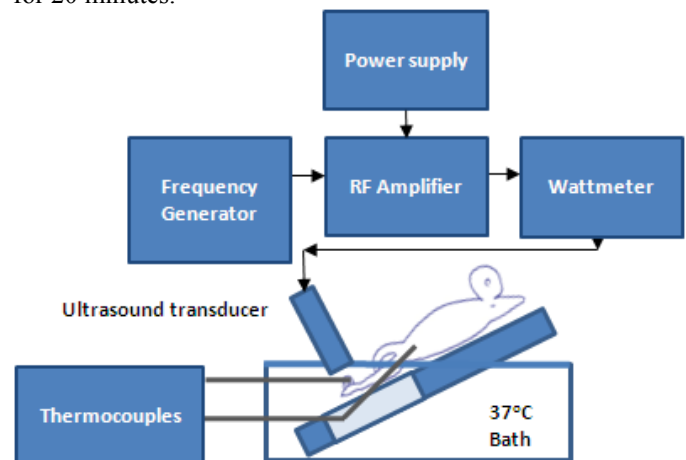
### HaT-DOX Formulation

This formulation was prepared as described previously [18-19]. A lipid mixture (15 mg) comprising 96 mol% DPPC and 4 mol% Brij78 (polyoxyethylene (20) stearyl ether) was dissolved in isopropyl alcohol and dried under a stream of nitrogen gas within a glass vial. The resulting thin lipid film was hydrated with 300 mM citric acid (1 mL). Liposomes were extruded through 100 nm polycarbonate membranes to prepare unilamellar ~110 nm diameter nanoparticles. The external buffer was exchanged with HEPES buffered saline (HBS, pH 7.4) using a Sepharose CL-4B column. The liposomes were then incubated with doxorubicin (DOX, 1:20 w/w ratio) for 90 min at 37°C. Unincorporated DOX was removed by dialysis. Encapsulation efficiency of DOX was >95%.

### Animal Model

Female BALB/c mice (aged 5-6 weeks, 18-20 g) were purchased from The Jackson Laboratory (Bar Harbor, ME). The experimental protocol was approved by the Animal Care Committee of the University Health Network (Toronto, Ontario, Canada) in accordance with the policies established in the Guide to the Care and Use of Experimental Animals prepared by the Canadian Council of Animal Care. The mouse mammary carcinoma cell line EMT-6 was a generous gift from Dr. Douglas Mahoney at the University of Ottawa. EMT-6 cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C with 5% CO<sub>2</sub>. EMT-6 cells (2 × 10<sup>5</sup> cells in 50 µl medium) were *s.c.* inoculated into the left footpad of the BALB/c mice (5 mice per group). The mice with developed EMT-6 footpad tumors (~7 days, 50-100 mm<sup>3</sup>) were anesthetized and secured to a tilted platform with an aperture located under the foot, and the platform was immersed in 37°C water, so that leg was immersed in the water bath. Just prior to anesthesia of each subject, 200 µL of HAT-DOX (10 mg/kg), free DOX (10 mg/kg) or saline (control) was injected *i.v.* A type K 25G thermocouple was positioned into the tumor, and a rectal thermocouple was placed to monitor body temperature. A

third thermocouple was placed in the water bath. All temperatures were displayed and stored on a datalogger (Omega HH309A). The ultrasound heating device consisted of a 12.7 mm diameter 3.9 MHz planar PZT4 crystal transducer (Boston Piezo-Optics, Bellingham, MA) housed in a Delrin casing. The most efficient operating frequency (3.912 MHz) was determined by measuring the reflection coefficient with an Agilent E5061A Network Analyzer. The transducer was driven by a 3.912 MHz continuous sine wave signal from an Agilent 33210A Function Generator, which was amplified by an in-house built amplifier kit. The electrical power from the amplifier was continuously measured by a WM-2 Wattmeter [Oak Hills Research, Aurora, CO], and initially the ultrasound output from the transducer was measured with an ONDA HNA-0400 and ONDA RFB2000 Radiation Force Balance. During the 20 minute heating experiments, 9.7 W (electric) was applied to the transducer producing 8 W (ultrasound) output, which is equivalent to 1.86 MPa. The transducer was immersed in a water bath over the footpad tumor (Figure 1), and the generator potential was adjusted to maintain the tumor temperature between 41-44°C for 20 minutes.



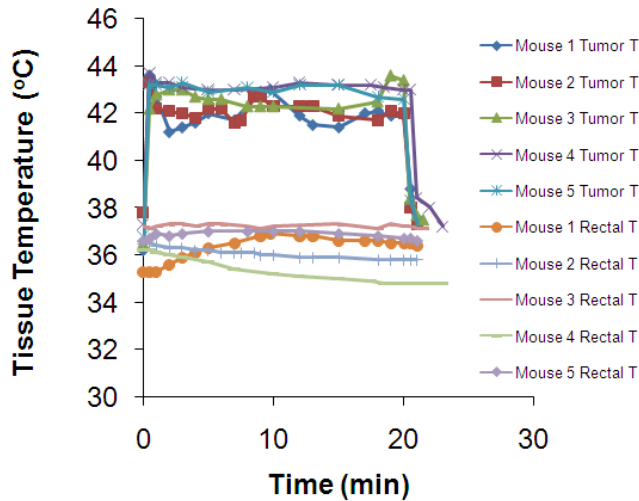
**Figure 1:** Schematic of treatment apparatus. Balb/c mice bearing footpad tumors were secured to a tilted platform, with the tumor-bearing foot positioned over an aperture.

Tumor dimensions and body weight before and after therapy were monitored to track the response to the respective therapies. Mice were sacrificed when humane endpoints were reached. Footpad tissue was collected from each subject, fixed in 10% formalin for 3 days, and mounted in paraffin for sectioning. Tissue sections were stained with H&E or immunohistochemically stained for apoptosis (TUNEL). In a second heating study, Balb/c mice were inoculated with EMT-6 cells in the breast fat pad to generate orthotopic breast tumors. Briefly, an incision was made at midline, the fatpad was located, and EMT-6 cells (2 × 10<sup>5</sup> cells in 50 µl medium) were gently injected. The incision sites were sutured, and mice were given time to recover and heal. Tumors were heated with ultrasound when they reached 50-100 mm<sup>3</sup>, and rectal and tumor temperatures were monitored.

### III. RESULTS AND DISCUSSION

The HaT-DOX formulation has previously been shown to enhance tumor uptake of DOX by 1.4-2 times compared to the LTSL formulation, with correlated enhancements to efficacy [18-20]. In this study, the HaT-DOX formulation was tested in conjunction with an ultrasound heating apparatus designed to confirm that ultrasound modalities are suitable for this technology. The apparatus (Figure 1) is suitable for small animal models (unlike clinical MRgFUS instruments), and permits us to screen formulation and cancer models more efficiently.

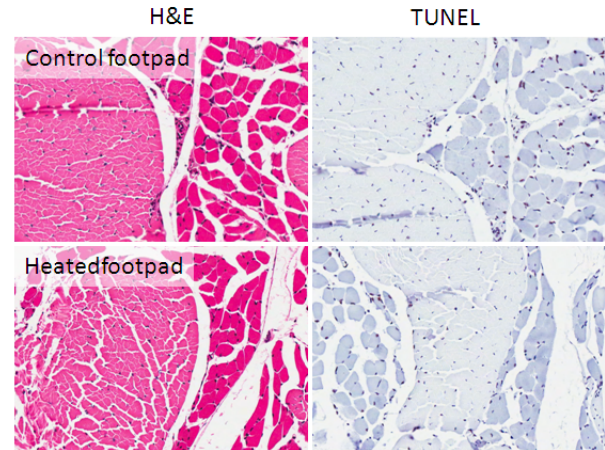
The induction of hyperthermia in mouse footpad tumors was rapid, with elevation of tissue temperature to  $42.6 \pm 0.6^\circ\text{C}$  (SD) within 30 seconds. When the transducer was energized, power output was adjusted between 5-8 W by manipulating the voltage from the function generator, to reach the  $41\text{-}43^\circ\text{C}$  setpoint. Adjustments to power output to maintain elevated temperatures were not necessary in the footpad model over the 20 min duration of testing (Figure 2), suggesting that steady-state heat transfer was established in the feet without delay. Body temperature was steady at  $36.3 \pm 0.7^\circ\text{C}$ , with one exception where a  $1.3^\circ\text{C}$  increase was observed.



**Figure 2:** EMT-6 tumor temperature and mouse body temperature were monitored. When the transducer was energized, tumor temperature instantaneously increased to  $41\text{-}43^\circ\text{C}$ , and body temperature remained stable at  $37^\circ\text{C}$ . Individual animal monitoring data is depicted to emphasize the range of response.

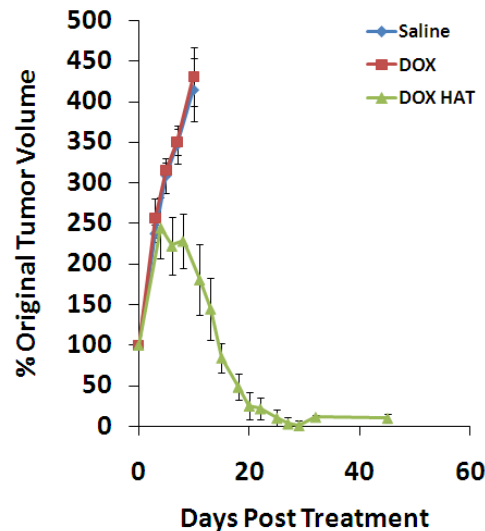
No obvious damage to the skin was observed post-heating, and by histology examination heated (non-tumor bearing) tissue was not affected by ultrasound heating, with no physiological alterations or indications of apoptosis/necrosis by TUNEL staining (Figure 3). In a second series of testing, orthotopic EMT-6 tumors were heated: in this model, elevation of temperature to  $41\text{-}43^\circ\text{C}$  was not as instantaneous (1-2 minutes), and throughout the course of heating (20 min), the output of the frequency generator had to be increased to maintain even heating. This particular observation was expected, as orthotopic tumors tend to be more heavily

vascularized [21-23], perfusion is known to influence heat transfer, and vascular parameters in turn are influenced by heating [13, 24].



**Figure 3:** Histology analysis of footpad tissues treated with ultrasound. 400X magnification. H&E stained sections indicate no alterations to tissue physiology, and TUNEL staining did not indicate any apoptosis (cell death).

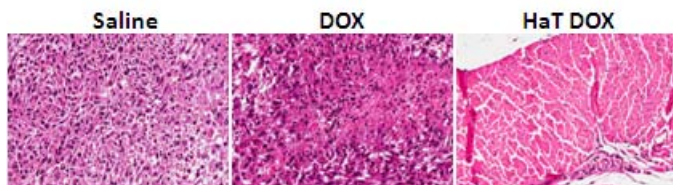
In the footpad model, post HaT-DOX therapy, treated mice exhibited swollen feet and redness in the tumor, a response to treatment consistent with doxorubicin therapy seen in other models. All mice (control, DOX, and HaT-DOX) were maintained for 1 week with buprenorphine treatments to manage inflammation. As the inflammation subsided, tumor regression in HaT-DOX treated mice was rapid, with complete regression being observed by 30 days, and the restoration of normal appearance and function of these feet, including gripping. Conversely, mice treated with DOX or saline rapidly reached tumor size endpoints (Figure 4). No therapeutic effect of DOX on the EMT-6 tumors was observed, even though this dose is the maximum tolerated dose in Balb/c mice [18-20].



**Figure 4:** Efficacy of HaT DOX / ultrasound therapy on EMT-6 breast tumor growth. HaT DOX (10 mg DOX/kg) completely inhibits tumor growth, leading to complete

remission of the tumor. Doxorubicin (10 mg/kg) has no effect on this tumor model. Error bars are SE, n=5.

By histology analysis (Figure 5), the physiology of HaT-DOX treated feet appeared normal, with no evidence of tumor tissue, although not all aspects of the original physiology seen in Figure 3 are present. However, the mice were treated when footpads were double their normal size, and already substantially infiltrated by tumor mass. The elimination of tumor tissue and restoration of function in these mice is remarkable in that light. As seen in the saline and DOX tumor cross sections, there is some evidence that free DOX was inducing necrosis, but these tumors are largely viable, which corresponds to the endpoints seen in Figure 4.



**Figure 5:** Histology analysis of treated footpad tumors. The disorganized pathology of tumor tissue is evident in the saline and DOX treated footpads, whereas the HaT-DOX treated mice exhibit no evidence of tumor tissue. 400X mag.

#### IV. CONCLUSION

Ultrasound induction of mild hyperthermia is an effective tool for triggering release of chemotherapeutic from HaT-DOX, a thermosensitive liposome formulation designed to enhance therapeutic outcomes in cancer treatment.

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