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ORIGINAL ARTICLE

Synergistic effects of thermosensitive liposomal doxorubicin, mild hyperthermia, and radiotherapy in breast cancer management: an orthotopic mouse model study

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Abstract

Liposome formulations of the cancer drug doxorubicin have been developed to address the severe side effects that result from administration of this drug in a conventional formulation. Among them, thermosensitive liposomal doxorubicin presents enhanced tumor targeting and efficient drug release when combined with mild hyperthermia localized to the tumor site. Exploiting the radiosensitizing benefits of localized thermal therapy, the integration of radiation therapy with the thermally activated liposomal system is posited to amplify the anti-tumor efficacy. This study explored a synergistic therapeutic strategy that combines thermosensitive liposomal doxorubicin, mild hyperthermia, and radiotherapy, using an orthotopic murine model of breast cancer. The protocol of sequential multi-modal treatment, incorporating low-dose chemotherapy and radiotherapy, substantially postponed the progression of primary tumor growth in comparison to the application of monotherapy at elevated dosages. Improvements in unheated distant lesions were also observed. Furthermore, the toxicity associated with the combination treatment was comparable to that of either thermosensitive liposome treatment or radiation alone at low doses. These outcomes underscore the potential of multi-modal therapeutic strategies to refine treatment efficacy while concurrently diminishing adverse effects in the management of breast cancer, providing valuable insight for the future refinement of thermosensitive liposomal doxorubicin applications.

Keywords Thermosensitive liposomes · Drug delivery · Hyperthermia · Doxorubicin · Nanomedicine · Radiotherapy

Abbreviations

DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
$DPPG_2$	1,2-dipalmitoyl-sn-glycero-3-phosphodiglycerol
$DSPE\text{-}mPEG_{2000}$	N-(carbonyl-methoxypolyethyleneglycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine
EPR	Enhanced permeability and retention
HCC	Hepatocellular carcinoma
HBS	HEPES buffered saline
HBSS	Hanks' balanced salt solution
H&E	Hematoxylin and eosin
HT	Mild hyperthermia
MSPC	1-stearoyl-2-lyso-sn-glycero-3-phosphocholine
RFA	Radiofrequency ablation
RT	Radiotherapy
ThermoDXR	Thermosensitive liposomal doxorubicin
TNBC	Triple negative breast cancer

Introduction

Despite continuing advancements in cancer treatment, breast cancer remains a challenging healthcare issue impacting millions of individuals worldwide [1]. Triple negative breast cancer (TNBC) is an aggressive subtype, representing around 15% of breast cancer cases. TNBC is characterized

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by a lack of expression of receptors commonly found in breast cancer including the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) [2]. The lack of these receptors limits the application of targeted therapies which includes drugs and antibodies that are directed at absent receptors, thereby narrowing treatment options in comparison to other breast cancer subtypes. Standard care for local disease is confined to surgery, chemotherapy, and radiotherapy. Approximately 30% of TNBC patients experience disease recurrence [3–6]. There is also a higher incidence of distant metastases for TNBC, compared to other breast cancer subtypes, which further complicates its management [5].

Chemotherapy continues to be an indispensable component in cancer treatment due to its potency in shrinking solid tumors and metastatic lesions. However, administration of chemotherapeutic agents, for example, doxorubicin, in conventional formulations is known to result in adverse effects owing to distribution of the drug to normal tissues [7]. Nanoparticles, such as liposomes, have been employed to deliver drugs to solid tumors with improved tumor targeting effect and thus reduced non-specific toxicity. For instance, doxorubicin has been encapsulated in nanosized pegylated liposomes (i.e., Doxil or Caelyx) to extend circulation lifetime and increase tumor accumulation through the enhanced permeability and retention (EPR) effect [8–10]. However, the efficacy of liposome formulations such as Doxil is limited due to variability in the EPR effect and incomplete release of drug at the tumor site [11, 12]. For example, Laginha et al. reported that, while a significantly larger amount of doxorubicin accumulates inside the tumor after intravenous administration of Doxil compared to conventional doxorubicin infusion, only half of the drug is available to enter the cell nucleus and bind to nuclear DNA [13].

Stimuli-responsive drug delivery systems offer a solution to overcome the reliance on the heterogeneous EPR effect among patients by releasing the payload in response to physical, chemical, or biological stimuli (e.g., temperature, pH, and enzymes) [14].

ThermoDox, a thermosensitive liposome formulation of doxorubicin, exemplifies the potential of a stimuli responsive system. In preclinical studies, ThermoDox demonstrated significant therapeutic benefits when combined with localized mild hyperthermia (HT) [15–17]. In these studies, ThermoDox in combination with a mild increase in tumor temperature (i.e., 39–43 °C) enabled the rapid release of doxorubicin in the tumor vasculature, delivering 12-fold more doxorubicin to the tumor region and significantly less drug to normal tissues including heart, liver, and kidney, compared to free drug administration [15, 18, 19]. The initial clinical development of ThermoDox, focused on combining it with radiofrequency ablation (RFA) to treat hepatocellular carcinoma (HCC). Unfortunately, this combination failed in two successive phase III trials known as HEAT (NCT00617981) and OPTIMA (NCT02112656) [20]. The clinical failure has been attributed to the low sensitivity of HCC to doxorubicin, the clinical dosing regimen, use of a suboptimal heating protocol along with improvements in the RFA technique [20, 21]. Current studies on thermosensitive liposome-based formulations of drugs have returned to a focus on use in combination with mild HT (i.e., T = 40–45 °C). There are two ongoing clinical trials on thermosensitive liposome formulations of doxorubicin: one evaluating ThermoDox in patients with relapsed solid tumors (NCT04791228) and another on doxorubicin encapsulated in $DPPG_2$-containing thermosensitive liposomes for treatment of soft tissue sarcoma patients (NCT05858710).

Mild HT can serve as more than a trigger for drug delivery, however its effects are temperature and time dependent [22, 23]. Mild HT can alter the tumor microenvironment. When applied locoregionally to the tumor site, it has been found to increase blood flow to the tumor as well as tumor vascular permeability [24]. This, in turn, can facilitate nanoparticle accumulation as well as drug penetration and improve the oxygen supply to the tumor [25]. Mild HT is also able to modulate immune cell function and thus amplify the anti-tumor immune response [26]. Clinically, HT has been shown to sensitize tumor tissue to radiation therapy (RT) through multiple mechanisms including an increase in oxygen levels and inhibition of DNA repair [27]. This boost in treatment effect that results in little to no increase in toxicity has led to growing interest in clinical use of mild HT as an adjuvant to RT [28, 29]. Studies have shown that this adjuvant strategy can also address resistance of tumor cells to RT, which is especially beneficial in the management of cancer recurrence in previously irradiated patients [30].

A recent clinical trial (NCT02850419) proposed to combine RT, mild HT, and heat-activated delivery of doxorubicin for the treatment of locoregional relapse of breast cancer. It was designed to exploit the immunomodulatory effect of mild HT, as well as its potential chemosensitization and radiosensitization capabilities, through the administration of a thermal dose in conjunction with ThermoDox and RT. The trial was unfortunately withdrawn following the failure of ThermoDox and RFA in phase III trials. Here, we build on the vision of this triple modality treatment approach in a preclinical model of murine TNBC. We hypothesize that the synergistic combination of ThermoDXR, a chemically equivalent formulation to ThermoDox, mild HT, and RT will enable a reduction in the doses of RT and chemotherapy required. This approach aims to mitigate treatment associated side effects, while still affording comparable or even greater therapeutic effect towards the primary disease

burden as well as distant metastases than a higher dose of a single treatment modality.

Methods

Preparation of ThermoDXR

Thermosensitive liposomes of doxorubicin were prepared using the thin film method previously reported by Dunne et al. [19]. Briefly, a thin film was obtained by evaporating solvent from a mixture of DPPC, MSPC, and DSPE-mPEG$_{2000}$ in chloroform at a molar ratio of 86:10:4. The thin film was hydrated through addition of sodium citrate buffer (pH 4.0, 300 mM) to yield a lipid concentration of 125 mM at 55 °C. The liposome suspension was passed through an extruder (Northern Lipids, Vancouver, Canada) with double-stacked 200 nm pore sized membranes under 200 psi nitrogen flow for 3 cycles, and then extruded through double-stacked membranes with a pore size of 100 nm at 400 psi for 10 cycles to obtain liposomes with a homogenous size distribution. Sodium carbonate buffer (pH 11.0, 500 mM) was used to adjust the pH of blank liposomes to 7.4. The liposomes were preheated to 35 °C prior to drug loading at a doxorubicin to lipid weight ratio of 1:20. Drug loaded liposomes were dialyzed against HEPES buffered saline (HBS, pH 7.4, 20 mM HEPES, 150 mM sodium chloride) at 4 °C overnight (MWCO 50 kD). Dialyzed doxorubicin-loaded liposomes were concentrated with a 50 kD MWCO tangential flow filtration module (Polysulfone MicroKros®, Spectrum, Rancho Dominguez, CA, USA) and sterilized using a 0.22 μm PES filter.

Characterization of ThermoDXR

ThermoDXR was diluted 100-fold in HBS buffer before size measurements were conducted using a Malvern Zetasizer Nano ZS (Malvern, WOR, UK). ThermoDXR diluted by the same factor in Milli Q water was prepared to measure the zeta potential with the same equipment. The phase transition temperature of ThermoDXR was determined by differential scanning calorimetry (TA Q100, TA Instruments, New Castle, DE, USA).

The concentration of encapsulated doxorubicin in the liposomes was measured by fluorescence as described previously [31]. Briefly, ThermoDXR liposomes were disrupted using Triton X-100. Standard doxorubicin solutions ranging from 10 ng/mL to 2000 ng/mL were prepared and mixed with the same amount of Triton X-100 as the ThermoDXR samples. Both liposome samples and standard solutions were excited at 470 nm and their fluorescence emissions quantified at 555 nm using a microplate reader (BioTek, Agilent Technologies, Santa Clara, CA, USA).

Cell culture and cytotoxicity assay

4T1 cells were obtained from ATCC (Manassas, VA, USA) and cultured in RPMI-1640 media containing 10% fetal bovine serum and 1% penicillin-streptomycin under 5% CO_2 at 37 °C. Adhered 4T1 cells were detached using 0.25% trypsin-EDTA solution before passaging. The acid phosphatase assay was used to assess the cytotoxicity of doxorubicin at 37 °C and 42 °C. Briefly, 4T1 cells were plated in 96-well plates at a density of 1000 cells per well and allowed to incubate for 24 h. Cells were then treated with the drug at varying concentrations. To investigate the impact of mild hyperthermia (HT), the first hour of drug exposure was conducted in an incubator with the temperature maintained at 42 °C. Following a total incubation period of 48 h, cells were incubated with phosphatase substrate (4-nitrophenyl phosphate, 2 mg/mL) for 2 h, after which 0.1 N NaOH was added to terminate the colorimetric reaction. The absorbance at 405 nm was then measured. GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA) was used to fit the data with dose-response curves and to calculate the IC50 values.

Tumor model

All animal studies were conducted in accordance with the guidelines of the Animal Care Committee at the University Health Network (UHN, Toronto, ON, Canada). An aliquot of 1×10^5 4T1 cells suspended in Hanks' balanced salt solution (HBSS) was injected into the mammary fat pad of female BALB/c mice at the age of 6–8 weeks to develop an orthotopic breast tumor model. Tumors were allowed to grow for nine days prior to treatment to reach approximately 100 mm^3. Mice were randomly allocated into different groups with 5–10 animals per group.

Tumor volume and animal body weight were measured every other day with an electronic calliper and digital mass scale, respectively. The largest diameter of the tumor was noted as length (l) and a perpendicular diameter was used as width ($_w$). Tumor volume was calculated according to the following formula $V = \frac{\pi}{6}\left(w^2\right)(l)$. Both tumor volumes and animal body weights were expressed as percentage of change compared to initial measurements on day 0 (i.e., first treatment day). Animals were euthanized and excluded once the tumor dimension was greater than 15 mm and/or the body weight loss $>20\%$ (i.e., ethical endpoint).

Mild hyperthermia and liposome treatment

Mice were anesthetized under isoflurane during treatments which were administered once per week for a total of three weeks (i.e., on day 0, 7, and 14 starting from the first treatment day, Fig. 1). The control and treatment groups included: (i) saline + HT; (ii) 4 Gy radiation + HT; (iii) 4 Gy radiation; (iv) 8 Gy radiation; (v) ThermoDXR 2 mg/kg + HT; (vi) ThermoDXR 3 mg/kg + HT; (vii) ThermoDXR 2 mg/kg + HT + 4 Gy radiation.

The HT treatment was conducted with a previously developed laser-based heating system [32]. Briefly, this setup included an illuminator containing a 763 nm diode laser transmitted through a 400 µm fiber and an enclosing cone built from highly reflective material to distribute light homogenously (± 15%) to the mouse tumor via an exit port. The illuminator was positioned above the tumor, with a temperature monitoring probe inserted into the tumor center. The power of the laser was manually adjusted within the range of 0.15–0.65 W/cm^2 to raise the tumor temperature to 42.5 °C and maintain that temperature for 25 min. Liposomes were administered through the mouse tail vein after 5 min of preheating and the heating treatment was continued for a further 20 min.

Radiation therapy

Animals were immediately transferred to the irradiator after HT treatment and underwent computed tomography (CT) scanning to guide subsequent radiotherapy (RT). Localized tumor irradiation was performed by an image-guided radiation system (SmART+, Precesion X-Ray Inc., Madison, CT, USA) at the STTARR Innovation Centre (STTARR, Toronto, ON, Canada). Animals were first imaged by CT scan to locate the tumor isocenter, followed by either 4 or 8 Gy irradiation using a copper filter and a 10–15 mm circular collimator depending on the tumor size. The irradiation was divided into two sessions, with the beam rotated 180° in the middle of the treatment to deliver half of the dose via the top of the tumor and half to the bottom, in order to avoid heterogeneous distribution of radiation within the tumor.

Determination of metastatic spread

All animals were euthanized with excessive CO_2 21 days after initial treatment. Lung tissue was collected immediately following euthanasia and fixed in 10% neutral buffered formalin. The right lung was embedded, sectioned, and stained with hematoxylin and eosin (H&E) by staff in the Pathology Research Program (PRP, Toronto, ON, Canada). The stained slides were scanned at 20X brightfield using Aperio AT2 system (Leica Biosystems, Concord, ON, Canada) at the Advanced Optical Microscopy Facility (AOMF, Toronto, ON, Canada). Lung images were processed with HALO® Image Analysis Platform (Indica Labs Inc., Albuquerque, NM, USA) to quantify the area of metastases and lung tissue.

Statistical analysis

Data are expressed as mean ± standard deviation (SD) unless otherwise stated. Statistical analysis was performed with GraphPad Prism 9.3.0 (GraphPad Software, San Diego, CA, USA). Multiple group comparisons were analyzed by one-way ANOVA test with Tukey correction and the significant difference between two treatment groups was compared by unpaired t-test. Power analysis of the lung metastasis results was conducted using the pwr package in R.

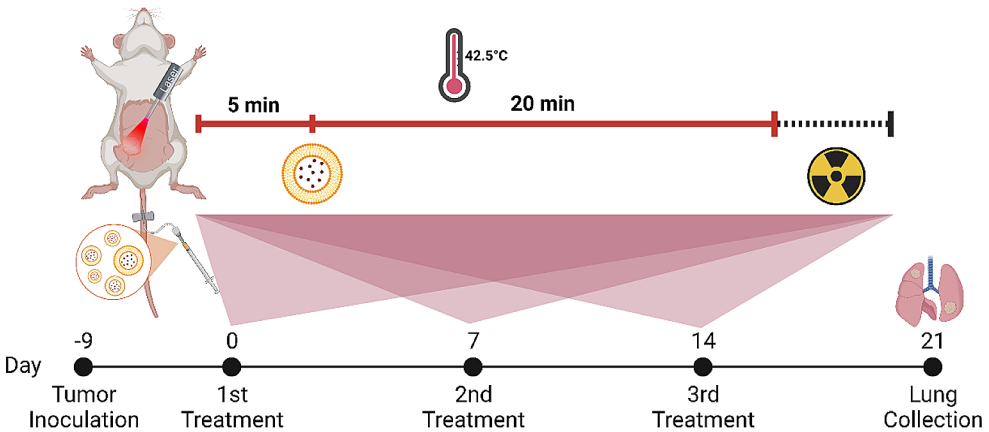

Fig. 1 Overview of animal treatment workflow to assess both the local and abscopal effects of combined thermosensitive liposomal doxorubicin (ThermoDXR), mild hyperthermia (HT), and radiotherapy (RT). Female BALB/c mice bearing orthotopic 4T1 breast cancer received 5-min of localized preheating (42.5 °C) to the primary tumor site prior to intravenous administration of ThermoDXR. HT treatment continued for an additional 20 min, followed by image-guided RT. This treatment regimen was administered once a week for three weeks. Animals were euthanized 7 days after the final treatment, and the lung tissue was harvested for histological analysis of metastases

Results

ThermoDXR characterization and drug cytotoxicity

The physicochemical properties of the ThermoDXR liposomes were found to be similar to those reported previously by our group [19]. Briefly, the liposomes had an average particle size of 94.5 nm with a PDI of 0.07 ± 0.01, indicating a homogenous size distribution. The zeta potential of the liposomes was found to be -26.2 ± 1.1 mV and the melting phase transition temperature (T_m) was 40.5 ± 0.1 °C, which is within the range of mild hyperthermia (HT).

The data from the cytotoxicity assay studies are included in Figure S1. Given the rapid release of drug seen from ThermoDXR in vitro upon exposure to temperatures in the mild hyperthermic range, it is postulated that in vivo the drug is largely released in the intravascular space and enters the tumor as free drug [19]. For this reason, the 4T1 breast cancer cells were treated with free doxorubicin in the absence or presence of HT exposure (i.e., 42 °C). No significant difference was found between the half maximal inhibitory concentration (IC_{50}) values for doxorubicin at physiological temperature and mild HT ($p = 0.30$).

Evaluation of primary tumor growth

The multi-modal treatment comprised of ThermoDXR combined with mild HT and RT, was evaluated in an orthotopic 4T1 mouse breast cancer model. This model is characterized by a primary solid tumor located at the mammary fat pad and metastatic lesions commonly found in the lung. Thus, use of this model enables assessment of treatment efficacy on the primary tumor as well as possible abscopal effects at distant sites. Saline + HT as well as RT + HT groups were employed as control groups to isolate independent variables. To select proper dosages of ThermoDXR and RT for the combination, the monotherapy of each modality at two different dosages (i.e., 2 or 3 mg/kg ThermoDXR and 4 or 8 Gy RT) was investigated. Free DXR was not included as one of the experimental groups, since the goal of the present study was to assess the therapeutic effect of the multimodal treatment at low dosages of each component therapy in comparison to that of high dose monotherapy.

As shown in Fig. 2 , mice treated with saline + HT had the greatest primary tumor growth, and the average tumor volume at the end of the study was more than 10-fold larger than the volume on the first day of treatment (1246 ± 118 vs. 101 ± 18 mm^3). Both thermosensitive liposomes and RT treatments delayed primary tumor growth in a dose dependent manner. Specifically, the therapeutic outcomes were significantly improved by the low dose treatments (i.e., 2 mg/kg ThermoDXR and 4 Gy RT) compared to the control group receiving saline + HT treatment ($p < 0.02$). The high dose RT (i.e., 8 Gy) further enhanced the effectiveness, leading to significantly slower growth of the primary tumor compared to lower dose RT ($p < 0.01$). Likewise, in comparison to animals treated with ThermoDXR at 2 mg/kg body weight and HT, mice that received 3 mg/kg ThermoDXR plus HT presented greater tumor growth inhibition with about 150% reduction in tumor volume change (603 ± 98% vs. 456 ± 76%, day 21 post initial treatment). Nonetheless, a significant difference was not found between these two groups ($p = 0.06$). Interestingly, the addition of HT to RT slightly stimulated the anti-tumor response to RT alone with this effect being observed following completion of the three weekly treatments (i.e., after treatment day 14, $p = 0.17$). The 4 Gy RT + HT group and the group of mice receiving 2 mg/kg ThermoDXR + HT had comparable tumor burdens

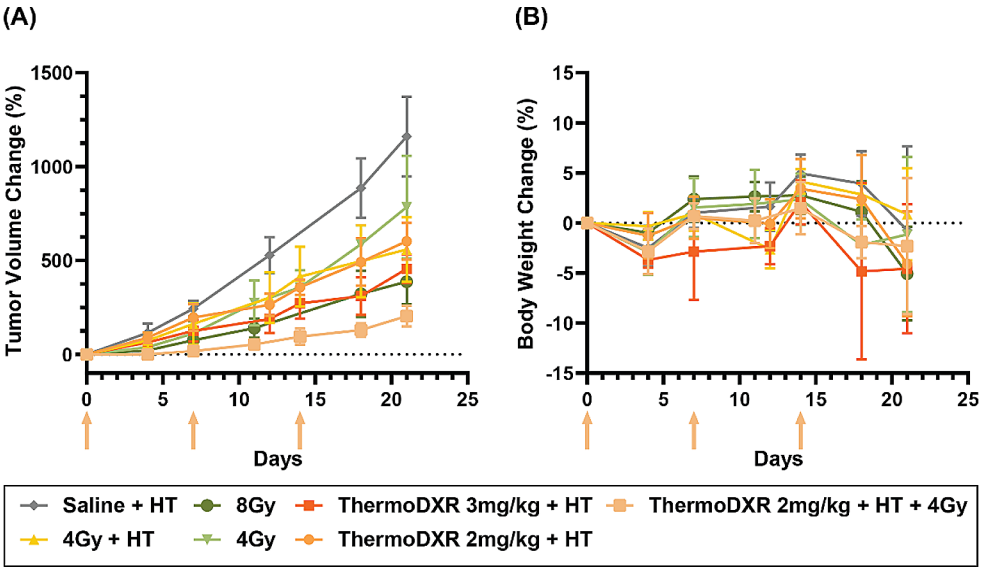

Fig. 2 Female BALB/c mice bearing orthotopic mouse 4T1 breast tumors were treated with mild hyperthermia (HT), 4 Gy radiation +/- HT, 8 Gy radiation, 2 or 3 mg/kg ThermoDXR + HT, and a combination of 2 mg/kg ThermoDXR + HT and 4 Gy radiation ($n \geq 5$). Three weekly treatments were administered on days 0, 7, and 14, as shown by the arrows below the x-axis. Tumor volumes (**A**) and animal weights (**B**) were compared to the original values on day 0 to illustrate the changes as a function of time. Dotted grid lines were added at y = 0 to represent the initial measurements of tumor volume and body weight on day 0. Data were expressed as mean ± SD

on day 21 post-treatment (Fig. 3 (A), 559 ± 172% vs. 603 ± 98%, $p = 0.67$).

The lower doses of both treatment approaches were incorporated into the multi-modal treatment group, where the animals were given 2 mg/kg ThermoDXR during HT treatment and exposed to 4 Gy RT within 9.0 ± 1.3 min after the HT (42.50 ± 0.31 °C) session was completed. The combination of all three treatments resulted in a mean tumor volume change that was notably reduced on day 21 in comparison to the groups receiving either 4 Gy + HT or 2 mg/kg ThermoDXR + HT ($p < 0.01$).

The combination of ThermoDXR (2 mg/kg) and RT (4 Gy) together with HT was also compared with the high dose groups of 3 mg/kg ThermoDXR + HT or 8 Gy RT alone to better understand the additional therapeutic benefits of the multi-modality treatment (Fig. 3). This triple combination provided superior inhibition of primary tumor growth by day 21 compared to the two high dose treatments (i.e., 3 mg/kg ThermoDXR + HT or 8 Gy RT) ($p < 0.03$). In addition, as shown in Fig. 3 (A), even though mice treated with 8 Gy RT experienced a relatively smaller change in primary tumor size than the group receiving 3 mg/kg ThermoDXR + HT, the difference was not statistically significant ($p = 0.52$).

Evaluation of treatment toxicity

Mouse body weight change was monitored as a proxy for toxicity of the administered treatments. In general, the animal weight fluctuated between − 5% and 5% of the initial weight between days 0 and 14 post the first treatment (Fig. 2). Both 2 mg/kg and 3 mg/kg ThermoDXR + HT treated mice showed a moderate decrease by less than 5% body weight after each treatment but recovered prior to the next treatment. In contrast, the animals undergoing saline + HT, RT + HT, or RT alone gradually gained body weight until the third treatment (i.e., day 14).

The highest animal body weights were observed on day 14 across all treatment groups. Body weight then began to fall, dropping continuously with the variability in each group increasing as a function of time, especially for the control group of saline + HT. At the final timepoint of this study (i.e., day 21; Fig. 3), only the group treated with RT at 4 Gy and HT exhibited a slight weight gain (0.9 ± 4.7%). Saline + HT and 4 Gy RT resulted in minimal weight loss, while administration of doxorubicin in thermosensitive liposomes generally led to more weight loss than 4 Gy RT. Interestingly, the addition of 4 Gy RT to the 2 mg/kg ThermoDXR + HT treatment reduced the weight loss caused by the ThermoDXR treatment. Moreover, the majority of mice treated with 8 Gy RT suffered a greater loss in weight compared with the low dose (i.e., 4 Gy) RT group (-5.1 ± 4.7% vs. -1.1 ± 7.7%, $p = 0.27$), indicating increased toxicity associated with this high dose of RT.

It is worth noting that the animal weight within each group was highly variable by the end of the study (Figure S2). For mice treated with saline + HT, 4 Gy RT, 2 mg/kg ThermoDXR + HT, or ThermoDXR + HT + RT, one animal in each group lost remarkably more weight than others in the group by day 21, experiencing a body weight loss exceeding 10% in the last measurement on day 21. In particular, an animal in the 4 Gy RT group reached − 19.5% body weight change, but 7 out of 8 mice in the same group maintained stable body weights. Similarly, 75% of the animals subjected

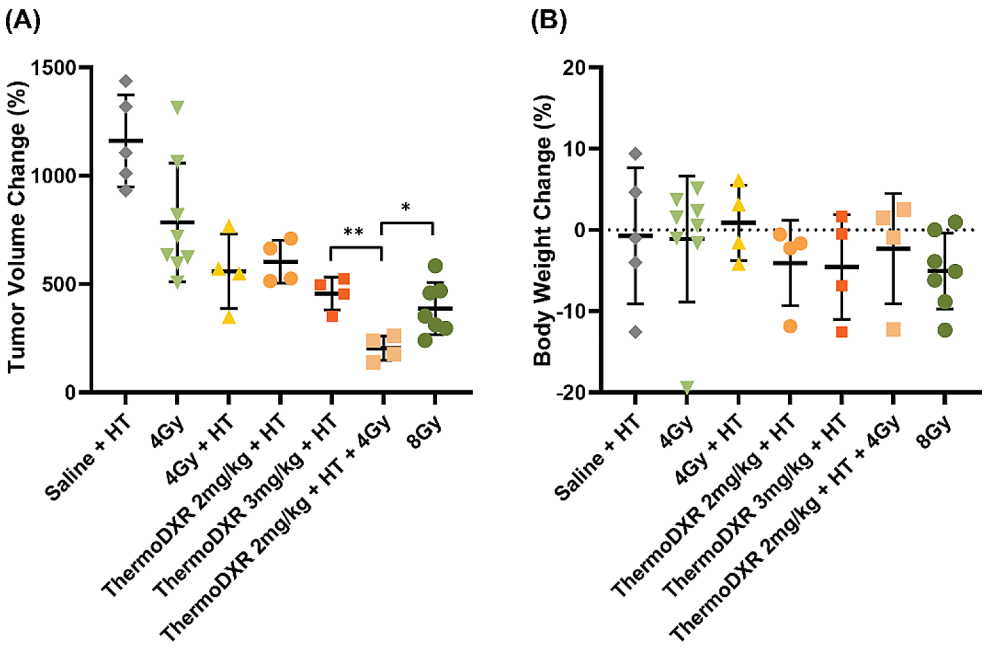

Fig. 3 Tumor volume increase (**A**) and animal body weight change (**B**) on day 21 post treatment for groups of mice bearing orthotopic 4T1 tumors. Data are shown as mean ± SD ($n \geq 4$). The combination of 2 mg/kg ThermoDXR with mild hyperthermia (HT) and subsequent 4 Gy radiation significantly inhibited the tumor growth in comparison to treatment with ThermoDXR + HT or radiotherapy at higher doses ($p < 0.03$) and appeared to result in a toxicity level similar to low dose radiation treatment alone ($p = 0.80$)

to the multi-modality treatment of ThermoDXR + HT and RT did not exemplify notable toxic effects in the last weight measurement on day 21, and minimal fluctuations in body weight were observed during the course of treatment.

Two mice reached ethical endpoints and were euthanized before the end of the study. Both low dose (2 mg/kg) and high dose (3 mg/kg) ThermoDXR + HT groups had one animal approaching endpoint on day 19 due to excessive primary tumor growth and more than 20% body weight loss, respectively. Additionally, two mice from the high dose (8 Gy) RT group and a mouse that received low dose (4 Gy) RT were found to be moribund on day 21.

Investigation of metastatic burden

To more thoroughly understand the therapeutic advantages of the multi-modal treatment and to discern the underlying causes of animal body weight loss observed by the study's conclusion, we assessed metastatic burden in the lung tissue on day 21 across different treatment groups. To quantify metastatic dissemination, we evaluated the area occupied by lung lesions in each section as a percentage of the total tissue area.

Significant variability in the degree of lung metastases was noted within each of the experimental groups (Fig. 4). For example, the region of the lungs occupied by metastases in the saline + HT treatment group ranged from 5.2 to 25.4%. This five-fold difference within one group was also found in the groups that received low dose (4 Gy) RT +/- HT suggesting heterogenous metastatic spread and varied response to treatment. A narrower interquartile range and lower lung metastatic area were observed in the group administered 3 mg/kg ThermoDXR + HT, in comparison to the three other aforementioned groups.

Three out of the four mice in the multi-modal treatment group exhibited a significant reduction in metastatic risk, which corresponded to a median percentage of lung metastasis area of 4.61%. Treatment with 8 Gy RT resulted in a notable reduction in distant metastases, marking the sole statistically significant reduction when compared to the saline + HT control group ($p = 0.02$). Nonetheless, there was no significant difference in the lung lesion area in mice receiving the triple combination versus high dose (8 Gy) RT ($p = 0.38$).

While the median % of lung metastases for the 2 mg/kg ThermoDXR + HT and RT treated group (4.61%) was lower than animals receiving saline + HT (12.04%), 4 Gy + HT (10.39%), and 2 mg/kg ThermoDXR + HT (14.05%), there was no significance noted between the triple combination and these other treatment groups. Due to the high degree of variability in metastatic spread within each treatment group, additional studies with larger treatment groups are needed to reach more robust conclusions. For instance, the power of the two-sample t-test between saline + HT group and the multi-modal treatment group was less than 30%. Increasing the sample size to 18 mice per group would help to identify if statistical differences actually exist and raise the power of the statistical test to 80%.

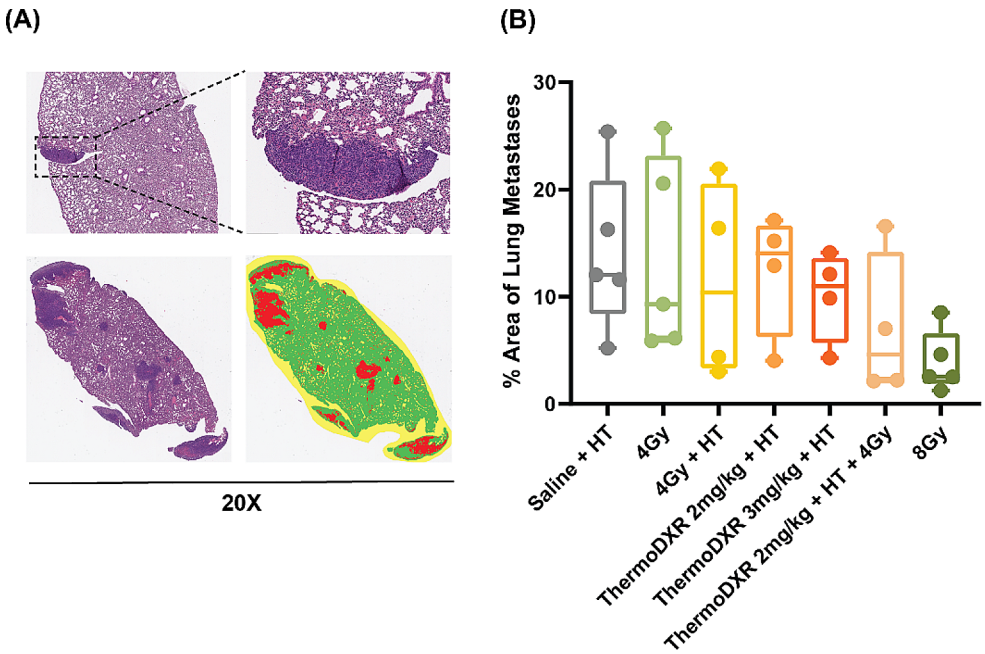

Fig. 4 Lung tissue was harvested from mice on day 21 following treatment with mild hyperthermia (HT), radiation (RT), ThermoDXR, or combinations thereof and subjected to H&E staining to visualize the metastatic lesions (i.e., with 3 non-consecutive sections analyzed per tissue sample). (**A**) Representative images of stained lung sections at 20X. The region of metastases, normal lung tissue, and background were labeled in red, green, and yellow, respectively. (**B**) The percentage area of lung metastases within the lung tissue is demonstrated for each treatment group. For each group, the middle line shows the median and the box represents the 1st and 3rd quartiles.

Discussion

Thermosensitive liposomes and mild hyperthermia in cancer treatment

In this study, we combined a hyperthermia-triggered liposome formulation of doxorubicin (ThermoDXR) with localized mild hyperthermia (HT) and radiotherapy (RT) to determine if the combination would enable an equivalent therapeutic effect to be achieved at a reduced dose of drug and RT. The results showed that a significant growth inhibition in locally advanced cancer can be achieved with combined treatment strategies of ThermoDXR, HT, and RT at dosages lower than that required for each monotherapy. The combination of the thermosensitive liposome formulation of doxorubicin and RT together with HT at reduced doses of drug and RT, respectively, not only induced a strong therapeutic effect but also reduced treatment related toxicities.

Thermosensitive liposomes are an advanced drug delivery system that can be thermally triggered to release their cargo within the tumor vasculature and overcome reliance on the heterogenous EPR effect. Lyso-thermosensitive liposomal doxorubicin, namely LTLD or ThermoDox, was among the most clinically advanced heat-activated nanomedicines and demonstrated its superiority over unencapsulated doxorubicin in preclinical studies [33–35]. Encapsulating doxorubicin in thermosensitive liposomes allows for efficient drug delivery to tumors undergoing HT treatment. This method increases the drug accumulation in the tumor compared to both free doxorubicin and non-thermosensitive liposomal doxorubicin (Doxil) when administered at a chemically equivalent dose [34]. Phase I trials of ThermoDox combined with either radiofrequency ablation or focused ultrasound induced HT yielded promising safety and feasibility results [35, 36]. While the clinical studies of ThermoDox did not reveal apparent cardiovascular toxicity of doxorubicin or the hand-foot syndrome commonly observed with liposomal doxorubicin treatment, adverse events including hematological toxicity closely mirrored those seen with intravenous administration of free drug [35]. This toxicity profile most likely arose from a proportion of the intravascularly released doxorubicin entering the systemic circulation. Although chemotherapy drugs are preferentially deposited in the tumor because of the rapid drug release in the tumor blood vessels and the consequent concentration gradient which drives drugs to enter tumor tissue, a tiny proportion of released drug is likely to be flushed out before completing the process. In fact, the systemic circulation of a small amount of drug released from thermosensitive liposomes at tumor vasculature has been confirmed in preclinical studies, for example, Dunne et al. detected unencapsulated doxorubicin at approximately 3%ID/mL blood collected via cardiac puncture 20 min after intravenous administration of a thermosensitive liposome formulation with HT [19].

Administration of PEGylated formulations can lead to the formation of anti-PEG antibodies which can result in accelerated clearance of the nanoparticles and reduced treatment efficacy [37, 38]. Though the seven-day interval between injections in the present work is relatively short for alleviating the acceleration of clearance [39], further investigation is required to identify if the treatment efficacy is altered due to multiple ThermoDXR treatments.

Although the cytotoxic effect of doxorubicin was not significantly increased when combined with one hour of mild HT exposure, it is important to note that the enhancement in cytotoxicity that can be caused by HT is influenced by a number of factors including the time and temperature of exposure as well as the cytotoxic threshold of the treated cells [25]. Moreover, HT is known to modulate the tumor microenvironment which is not captured by our in vitro studies conducted in monolayers of tumor cells alone. As a result, the effect of HT observed in the cytotoxicity assay may not fully recapitulate the effect of HT on efficacy in vivo.

Combination therapies that include radiation

Doxorubicin and RT are considered a synergistic combination due to their complementary mechanisms of action [40–42]. Although doxorubicin is highly active against breast cancer, the dose of doxorubicin is commonly reduced for administration with radiation therapy [43]. This is due to the fact that the heart is inevitably exposed to irradiation during RT of the left breast which can aggravate the cardiotoxicity induced by doxorubicin [44–47]. The replacement of free drug with pegylated doxorubicin can improve the efficacy associated with chemoradiation therapy and avoid cardiac tissue damage [40].

Pre-irradiated cancer patients may not respond well to repeat RT for the treatment of local recurrences [48, 49]. A solution to overcome the radioresistance involves combining RT with a radiosensitizer such as HT. Although thermal therapy is not widely implemented in standard cancer treatments, it is commonly recognized as an approach with minimal clinical toxicity and is being increasingly investigated in clinical trials to determine its potential as a novel treatment option [29, 50]. A retrospective analysis of repeat irradiation of patients with locally recurrent breast cancer suggested that the addition of HT to hypofractionated RT has the potential to improve the RT response as the observed increase in complete response rate was nearly statistically significant ($p = 0.08$) [51]. More promising clinical evidence has emerged from a study which examined the integrated treatment of HT at 43 °C and low dose RT, with an

extremely short time interval of 1–4 min between the two modalities [30]. In this study, 61% of breast cancer patients with sizable recurrent tumors showed complete tumor remission, and 59% of them successfully maintained the complete response throughout their lifetimes. More importantly, only grade 1 toxicity was found in the participant cohort.

Thermochemoradiotherapy has also been explored in various cancer types. Clinically, the triple combination of external beam RT, HT, and chemotherapy is viable and effective for cervical cancer and significantly increases the 5-year overall survival to 81.9% compared to standard chemoradiation treatment [52, 53]. Thermochemoradiotherapy has also been shown to result in a favorable complete response rate (80%) in breast cancer patients with chest wall recurrence, wherein 76% of the patients achieved 1-year local progression free survival [54]. These studies have largely employed unencapsulated chemotherapy drugs including cisplatin, capecitabine, paclitaxel, and vinorelbine. Doxorubicin has been encapsulated in non-thermosensitive liposomes for investigation of this triple combination approach in locally recurrent breast cancer [55]. The inclusion of nanomedicine was proven to be well tolerated. All participants responded to the treatments with 20% of them showing no evidence of residual tumor. Separately, in a preclinical cell study, Besse et al. deemed ThermoDox a radiosensitizer delivery platform and confirmed that combined ThermoDox, HT at 43 °C, and RT was able to enhance irradiation effects in human fibrosarcoma cells [56].

Before translating the concept of thermochemoradiation to either the preclinical or clinical setting, it is necessary to identify the sequence of HT and RT. Whether HT is placed as the first treatment depends on the mechanisms of interaction between different treatment components, as well as the capacity of the preclinical or clinical facility. In our circumstance, HT must be integrated with administration of the thermosensitive liposomes to ensure rapid drug release and tumor targeting. Unfortunately, concurrent administration of HT and RT was not feasible due to limitations within our facility. Our sequential treatment workflow involved preheating the orthotopic tumor with localized HT for 5 min prior to intravenous injection of the liposomal formulation (Fig. 1). The localized HT was continued for another 20 min and then followed by an immediate X-ray irradiation to the disease areas. In this way, HT was able to prime both the cancer cells and tumor microenvironment to gain reinforced effect of DNA damage or cell killing induced by doxorubicin and RT. Moreover, a relatively short time lag between HT and RT has been reported to be associated with better treatment outcomes, no matter if HT is conducted before RT or after [57]. Concurrent treatment of HT and RT is expected to achieve the greatest thermal enhancement, however, in reality this can be challenging to execute [58]. Therefore, it is critical to shorten the time interval between two treatment approaches as much as possible to maximize therapeutic benefits. In the present study, we managed to apply the RT within 10 min after completion of HT to maximize the treatment effect.

Abscopal effect

The potency of RT and its combination is often broadened, from localized impact to the inhibition of distant metastases. Irradiation localized to the primary tumor can elicit indirect regression of lesions outside the treatment field which was first conceptualized as the abscopal effect by Mole et al. [59]. While the abscopal phenomenon is aspiring, the clinical observation of this effect is rare and dependent on the inherent immunogenicity of the disease entity [60].

The multi-modal combination treatment strategy has the potential to leverage all three elements (i.e., RT, chemotherapy, and HT) to induce enhanced abscopal effects. RT is indispensable for eliciting the anti-tumor response outside the treatment field. As illustrated in our results, high dose RT at 8 Gy becomes the only treatment method that significantly diminishes metastases development in the 4T1 tumor bearing mice, while the liposome treatments resulted in limited control over metastatic progression.

Evolving understanding about the mechanisms behind the abscopal effect has been directed to immune activation, specifically immunogenic cell death [61, 62]. The dying cancer cells secrete or expose damage-associated molecular patterns which promote antigen uptake by dendritic cells and sequentially activate a series of T cell responses [63].

Interestingly, chemotherapy drugs including doxorubicin are also found to be potent immunogenic cell death inducers [64, 65]. Therefore, the RT induced abscopal effect may be amplified by combining with doxorubicin which participates in the overlapping immunological pathway. This speculation has been confirmed by a study showing significantly enhanced abscopal effect following the addition of a single dose of Doxil to a combination of RT/αPD-1 [66]. The enhancement was found to be related to an increasing number of dendritic cells and $CD8^+$ T cells which is an essential hallmark of immunogenic cell death.

In addition, HT is known to boost the immune response by modulating the tumor microenvironment. Specifically, HT elevates the immunogenicity and enhances immune cell infiltration into the tumor [26, 67, 68]. As a result, HT is increasingly used in combination with RT, chemotherapy, or immune checkpoint blockade therapy to improve treatment of peritoneal metastases and bone metastases [69–71]. Contrary to the stimulated immune response, there is also evidence showing that HT tends to increase the number

metastatic lesions and the effect varies depending on temperatures and length of the HT treatment [72]. These contradictory results underscore the need for further investigation of the systemic effects of HT.

Last but not least, thermally triggered nanomedicine alone is found to exert systemic effects in the present metastatic breast cancer model as well as bilateral tumor models [73, 74]. Besides the drug induced anti-tumor immune response, such an effect has also been attributed to the systemic circulation of drug molecules released in the tumor vasculature.

Cancer cells metastasize through a highly complex cascade which is impacted by not only the metastatic cells themselves but also the neighboring extracellular matrix [75]. Thus, the metastatic potential of a malignant tumor can be heterogenous [76]. Employing the 4T1 mammary tumor model allows us to clarify the systemic impact caused by the multi-modal treatment. However, as observed, it is important to note that the natural variability in metastatic potential can complicate the interpretation of the results. Oei et al. counted the number of lung metastases in luciferase-transfected 4T1 tumor bearing mice that received the combination treatment of RT, HT, and immune checkpoint inhibition therapy [72]. Given the great degree of variability in the number of lesions found in all cohorts, the authors highlighted the importance of taking lesion size into consideration. Our study on the lung metastasis area reveals heterogeneous spread of metastases in agreement with published results, highlighting the variability associated with individual metastases progression.

In conclusion, our study demonstrates that the combination of ThermoDXR, localized mild HT, and RT significantly reduces the disease burden at the primary tumor site more effectively than the use of ThermoDXR with HT or RT alone at higher doses. Additionally, this combination therapy allows for lower doses of chemotherapy and RT, thereby minimizing associated toxicities. Notably, this approach also enhanced the abscopal effect on lung metastases compared to low-dose monotherapy, though further research is needed in this area. As the inaugural preclinical investigation into the combined use of heat-activated drug delivery and RT in breast cancer, our findings provide valuable insights into how this multi-modal strategy can achieve both local disease control and systemic benefits.

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Author contributions Xuehan Wang: conceptualization, methodology, investigation, analysis, writing-original draft, writing-revision, and visualization. Christine Allen: conceptualization, writing-review and editing, supervision, funding acquisition.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval All animal studies were conducted in accordance with the guidelines of the Animal Care Committee at the University Health Network (UHN, Toronto, ON, Canada).

Conflict of interest The authors have no relevant interests to disclose.

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