MODULATED ELECTRO-HYPERTHERMIA ENHANCES THE EFFICACY OF ANTI-CANCER DRUGS IN MICE WITH TRIPLE-NEGATIVE BREAST CANCER

PhD thesis

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1. Introduction

Triple-negative breast cancer (TNBC) is a heterogeneous breast cancer subtype that represents 10-15% of all breast cancer cases. Due to the lack of expression of estrogen receptor (ER), progesterone receptor (PR), and the human epidermal growth factor receptor (HER-2), hormone and targeted therapies are not effective. Thus, the limited treatment options of TNBC necessitate the need for alternative treatments.

Modulated electro-hyperthermia (mEHT) is an advanced option in the mild hyperthermia field, applying 13.56 radiofrequency electromagnetic current generated by a capacitive coupling set-up between two electrodes. mEHT is approved for cancer therapy in several countries, exhibiting promising results in cancer response with no serious side effects in different cancer types. The cancer-specific damage of mEHT is based on the difference in bioelectrical properties between the cancerous and healthy tissues. This bioelectrical difference results from the higher aerobic glycolysis of cancer cells that causes higher ion and lactate levels and thus elevates the electric conductivity of the cancer tissue. These factors result in

the selective absorption of the energy of an electromagnetic field by the cancer tissue.

mEHT can enhance the delivery and anti-cancer effects of cancer therapeutics by improving tumor blood flow (TBF) and exerting direct cancer cell-killing effects. Effective delivery of therapies to cancerous tissue relies heavily on sufficient TBF. Thus, mEHT can synergistically improve the clinical outcome of the anti-cancer agents.

Doxorubicin (DOX), is a frequently utilized anti-cancer agent for the treatment of various cancer types, including TNBC. Despite its efficacy, the utilization of DOX is constrained by the occurrence of adverse effects, notably cardiotoxicity and myelosuppression. Two liposomal formulations of DOX have been approved for clinical application: PEGylated (PLD) and non-PEGylated liposomal DOX. Although the authorized formulations decreased the cardiotoxicity, their therapeutic effectiveness is not superior to conventional DOX due to their dependence on the enhanced permeability and retention (EPR) effect.

Lyso-thermosensitive liposomal DOX (LTLD, Thermodox®) presents a promising approach to overcome the limitations of the approved liposomal formulations due to an ultra-fast release of DOX at temperatures > 39.5 °C (≈80% within 20 s). However, following successful phase I and II clinical trials, two phase III clinical trials failed to reach the primary and secondary endpoints in hepatocellular carcinoma (HCC) patients. Thus, the cancer-selective heating of mEHT might introduce it as a more efficient induction of DOX release from LTLD within the tumor.

Digoxin, a cardiac glycoside, serves as a medication for various heart problems such as congestive heart failure, and cardiac arrhythmias (3). Several studies have demonstrated anti-cancer effects of digoxin in different cancer models. However, the in vivo efficacy of digoxin has not been investigated before in TNBC. Thus, in this study, we investigated the anti-cancer effects of digoxin in combination with mEHT in TNBC mouse model.

2. Objectives

In the studies described in the present dissertation, we investigated the effects of mEHT on the delivery and in vivo efficacy of agents used in cancer therapy. In particular, we evaluated whether mEHT can:

- Enhance the cancer-specific delivery of DOX-encapsulated in LTLD
- Improve the cancer cell death of DOX-encapsulated in LTLD compared to free DOX and PLD
- Synergistically enhance the tumor growth inhibition of digoxin

3. Methods

Orthotopic breast tumors were induced in female BALB/c mice by subcutaneous injection of 1×10^6 4T1 cells in 50 μL Phosphate-Buffered Saline (PBS) into the fat pad of the 4th mammary gland under isoflurane anesthesia (5% isoflurane for induction and 1.5–2% isoflurane to maintain anesthesia). On the eighth day after inoculation, the tumor size was measured using ultrasound (US) by visualizing the tumor in two perpendicular planes and measuring the length and width of the tumor (a,b). The (c) diameter was averaged from the depth of the tumor measured in the two positions. In addition, the digital caliper was used to measure three perpendicular diameters (a, b, c) of the tumor. The tumor volume (V) was calculated by the following formula: $V = (a \times b \times c \times \pi)/6$.

We performed three experiments. The aim of the first experiment was to compare the effects of mEHT+LTLD to the other DOX formulations. Thus, 8 days after the implantation of 4T1 cells, the mice were randomly divided into 7 groups based on tumor volume and body weight: sham; mEHT; DOX; PLD; mEHT+DOX; mEHT+PLD; and mEHT+LTLD (n = 5–6). As LTLD is used in the

clinic only in combination with hyperthermia, we omitted the LTLD+sham group from this study.

DOX, PLD, and LTLD were injected at a dose of 7.5 mg/kg into the retro-orbital venous plexus, and the sham and mEHT groups received equivalent amounts of 0.9% saline. Based on our pilot study, LTLD was administered slowly over 3 min to avoid any hypersensitivity reactions. Immediately after the administration of the drugs, mEHT was performed for 30 min. The treatment was repeated 3 times at 72 h intervals. During the study, mice were monitored by measuring the tumor volume and body weight. Changes in body weight were used as an indicator of systemic toxicity. Mice were sacrificed 24 hours after the last treatment by cervical dislocation, and the tumor tissues were resected. One-half of the tumor was placed in a 4% formaldehyde solution and transferred for histological processing. The other half was stored in liquid nitrogen at −80 °C for molecular analysis.

The purpose of the second experiment was to follow the growth of the tumors for 5 days after the third treatment to demonstrate when the tumors stop shrinking. Based on the results of the first experiment, the mice were randomized

into three groups: sham+vehicle (n=5), mEHT+PLD (n= 7), and mEHT+LTLD. We followed the same protocol as in the first experiment.

In the third experiment, we tested the efficacy of mEHT and digoxin combination in the same mouse model. However, mEHT treatment was performed four times with 48-h interval. In addition, 2 mg/kg dose of digoxin was administered daily by intraperitoneal injection. Mice in mEHT and sham groups received equivalent amounts of saline. Tumor size was measured by a digital caliper.

In vivo imaging system (IVIS) was used to measure DOX accumulation in the tumor at 1 and 24 h after the administration of DOX, PLD and LTLD with or without mEHT.

Hematoxylin-eosin (H&E)-stained sections were scanned and analyzed using Case Viewer image analysis software. We annotated the damaged areas from the viable areas under high digital magnification in the case of each scanned tumor sample. Tumor destruction ratio (TDR) % was calculated by dividing the damaged area by the whole tumor area. Furthermore, pro-apoptotic and anti-proliferative effects of the treatment were evaluated using

western blot and immunohistochemistry (IHC) of cleaved-caspase3 (cC3) and Ki67, respectively.

Statistical analysis**:** Data are shown as mean ± SEM. GraphPad Prism software (v.6.01; GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis. Changes in tumor volume, body weight and DOX accumulation in the tumor were compared between groups by two-way ANOVA and Tukey's post-hoc test. The one-way ANOVA, followed by Tukey's post-hoc test, was used to analyze changes in tumor weight and expression of cC3 and Ki67. The null hypothesis was rejected if * $p < 0.05$.

4. Results

Upon tumor removal, tumors were the largest in sham+veh and sham+DOX groups. Sham+PLD, mEHT+veh and mEHT+DOX tumors were smaller, and mEHT+PLD and mEHT+LTLD tumors were the smallest. The mEHT+LTLD tumors were smaller than the mEHT+PLD tumors, however this difference was not statistically significant. mEHT+LTLD was the only treatment that significantly reduced tumor weight compared to mEHT+DOX.

A separate experiment was conducted to compare the tumor growth inhibition of PLD and LTLD for 5 days after the termination of the treatments. mEHT+LTLD was significantly more effective in inhibiting tumor growth than mEHT+PLD. Based on the ultrasound recording this difference was already visible after the second treatment. Tumors treated with (mEHT+LTLD) or (mEHT+PLD) stopped growing already after the second treatment and did not regrow during the following 5-day observation period after the last treatment.

IVIS did not detect any DOX autofluorescence in tumors treated with sham+DOX, sham+PLD, mEHT+DOX, and

mEHT+PLD one hour after the treatment. Conversely, a strong DOX autofluorescence signal was observed in tumors treated with mEHT+LTLD, indicating the highest accumulation of DOX in the tumor at the same time point. The TDR, estimated as the percentage of the damaged area to the whole tissue area in H&E sections, demonstrated that TDR in the mEHT+LTLD group was significantly higher than in all other groups except mEHT+PLD. Although there was a slight difference, mEHT+LTLD did not result in a significantly different TDR as compared to mEHT+PLD.

The IHC of cC3 exhibited that the most intensive cC3 staining was observed in mEHT+LTLD-treated tumors. In line with IHC findings, western blotting of cC3 showed that cC3 expression in mEHT+LTLD-treated tumors was significantly higher than in tumors from all other groups.

The IHC of Ki67 proliferation marker demonstrated that the mEHT+LTLD group had the lowest number of Ki67+ nuclei. Similarly, western blotting analysis of Ki67 revealed that tumors treated with mEHT+LTLD displayed the lowest Ki67 expression.

Monitoring of mice's body weight showed a significant decrease in body weight observed in all mice treated with any DOX formulation (DOX, PLD, or LTLD). These groups exhibited parallel body weight loss, and there was no significant difference in the kinetics of the body weight loss between them.

In the mEHT+ digoxin study, digoxin monotherapy did not influence tumor volume during the study or at the end of the study compared to sham+saline. However, by the end of the study, mEHT+digoxin-treated tumors were significantly smaller than tumors in all other groups. Body weight was not reduced significantly in any of the study groups, suggesting the lack of severe toxicity.

5. Conclusions

1. mEHT can be used as a source of hyperthermia to activate thermosensitive nanoparticles such as lyso-thermosensitive liposomal doxorubicin (LTLD)
2. mEHT+LTLD is a novel, EPR-independent approach for DOX delivery to the cancer
3. mEHT accelerates tumor delivery of DOX from LTLD-encapsulated DOX
4. mEHT augments cancer destruction induced by different formulations of DOX, especially LTLD
5. The synergism between mEHT and DOX is mediated by inducing apoptosis and inhibiting proliferation of cancer cells
6. mEHT improves the anti-cancer effect of digoxin in vivo
7. mEHT+digoxin is a safe and novel treatment option for TNBC

6. Bibliography of the candidate's publications

Publications related to the thesis

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