ANTITUMOR EFFECTS OF MODULATED ELECTRO-HYPERTHERMIA IN 4T1 TRIPLE-NEGATIVE BREAST CANCER MODELS

PhD thesis

Lea Danics PharmD

Doctoral School of Theoretical and Translational Medicine

Semmelweis University

Supervisor:	Péter Hamar, MD, DSc
Official reviewers:	Attila Marcell Szász, MD, PhD Gábor Pozsgai, MD, PhD

Head of the Complex Examination Committee: Janina Kulka, MD, DSc

Members of the Complex Examination Committee: Mihály Újhelyi, MD PhD
Dániel S. Veres, MD, PhD

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1. Introduction

Breast cancer is one of the leading causes of cancer-related death among women. One of the most aggressive type of breast cancers is triple-negative breast cancer (TNBC), which accounts for about 15% of all breast cancers and has a very poor prognosis. Triple-negative breast cancer is a special subtype of breast cancers, as it does not express any estrogen-, progesterone-receptors or human epidermal growth factor receptor 2 (HER2), thus targeted therapies cannot be used. For those patients, who are diagnosed with TNBC the only approved systematic treatment option is the highly toxic and non-selective chemotherapy. Further steps are needed to identify new therapeutic possibilities.

For modeling and investigating the human TNBC disease in preclinical settings, several small animal models were established. One of these is the 4T1 mouse model, which is widely used in preclinical research. 4T1 cells were isolated from a spontaneously arisen 410.4 mammary carcinoma of a female BALB/c mouse by Fred Miller *et al.* and selected from the other three sublines (4T07, 168FARN and 67NR) based on its natural resistance to 6-thioguanine. 4T1 cells have a triple-negative phenotype (no expression of estrogen-, progesterone-receptors or HER2) and they are strongly tumorigenic and invasive with a very high metastatic ability to lymph nodes, blood, liver, lung, brain and bone. Inoculating 4T1 cells into syngeneic BALB/c mice (isograft) provides a suitable, immunocompetent model for triple-negative breast cancer,

since the molecular and pathophysiological characteristics are very similar to those of the human disease.

Hyperthermia in oncology refers to the use of therapeutic heat energy to treat various malignancies. This treatment modality is considered as a complementary oncology treatment and is always used in combination with other therapies, such as chemo-, radio- or targeted and other biological oncotherapies. The intake of the applied energy usually results in an elevated intra- and peritumoral temperature at the treated site in the range of 40-48 °C, which is maintained for one or more hours. According to the dimensions of the treated site, there are different clinical applications of hyperthermia: local, regional or whole-body hyperthermia.

Modulated electro-hyperthermia (mEHT) is a novel complementary loco-regional hiperthermic antitumor therapy, which applies alternating electromagnetic field to communicate energy to the tumor. It can induce highly selective heating of tumors by capacitive-coupled energy transfer, where the capacitor is the tumor itself. The mechanism of this selective heating is based on the altered complex electric properties of the tumors compared to normal tissues. mEHT is a unique capacitive electromagnetic treatment modality, as it induces not only thermal but also non-thermal effects in the tumor by using amplitude modulation of the 13.56 MHz radiofrequency. Thermal effects are direct consequences of the elevated temperature and they are absolutely

temperature-dependent (e.g. altered blood flow, hypoxia, protein denaturation, cellular damage etc.). Non-thermal effects of modulated electro-hyperthermia are mediated by the alternating electromagnetic field. The non-thermal effects are mainly frequency-dependent and result from the interaction of the biological substance with the RF-current (e.g. polarization, rotation and electrophoresis of bioactive molecules, altered membrane fluidity and permeability etc.)

Heat shock response (HSR) of living organisms is an ancient and universal mechanism, which refers to a complex molecular defense process activated upon cell stress. Heat shock – or more generally cell stress – induces disturbance in protein homeostasis, cytoskeletal structure, nuclear processes and brings about changes in the cell membranes. These morphological and functional changes trigger the heat shock response, which strictly speaking is a rapid and transient gene transcription program in order to protect the organism against damage. The main effectors of the heat shock response are the so-called heat shock proteins, like Hsp70 and Hsp90, which are molecular chaperons and, as such, they are able to recognize and refold or assist in eliminating misfolded proteins. When cell stress occurs, misfolded proteins induces the transcription of Hsps, which restore normal protein homeostasis. The transcription of Hsps is initiated by the transcription factor heat shock factor-1 (Hsf-1). Activation of the heat shock response provides protection for the tumor cells against the heat-, radiation- or

chemotherapy-induced damage, and thus it attenuates the efficiency of antitumor treatments. It has been already described that overexpression of the heat shock proteins is one of the most important survival mechanisms of cancers, which helps them to resist therapies. Inhibition of this defense mechanism could sensitize the tumor cells to hyperthermia or other antitumor therapies.

The experimental work summarized in my thesis aimed to optimize mEHT treatment and investigate its effects in TNBC by using a preclinical mEHT treatment device on a mouse model of TNBC.

2. Objectives

The studies summarized in this thesis are aimed to investigate the macro- and micromolecular effects of mEHT in the 4T1 triple-negative breast cancer models both *in vitro* and *in vivo* by focusing on:

- the development of a rodent modulated electro-hyperthermia device for orthotopic mouse breast cancer treatment *in vivo*
- the tumor cell killing and tumor destructive effects of repeated mEHT *in vivo*
- the apoptosis-inducing effects of repeated mEHT *in vivo*
- the temporal changes in mEHT effectiveness *in vivo*
- the heat shock response and its dynamics after repeated mEHT treatments *in vivo*
- the synergistic effects of mEHT and heat shock response inhibitors (quercetin and KRIBB11) *in vitro*

3. Results

We introduced a newly improved preclinical mEHT device LabEHY-200 and the ergonomic pole electrode optimized for treating orthotopic mammary tumors in the inguinal region of the mice. Our experiments demonstrated that the tumors could be heated up effectively and selectively by this improved rodent mEHT treatment setup thanks to the more accurate skin-electrode contact and improved tissue coupling. The treatments became more efficient and standard even at lower power, and the more focused treatments prevented heat-related side effects. Better focus and coupling reduced the variability in temperature and power during treatments. The rectal temperature remained within the normal range (T_{rectal} = 37.03 ± 0.61 °C) and treatments became local and reproducible.

The treatments had a significant tumor cell-killing effect *in vivo* testified by In Vivo Imaging (IVIS). To demonstrate this, we inoculated firefly luciferase transfected 4T1 cells orthotopically into mice, then we monitored the amount of viable tumor cells after mEHT treatments in the presence of D-luciferin. The viable 4T1 tumor cells, which stably express the luciferase enzyme, cleave luciferin, which is accompanied by fluorescent light emission. The intensity of detected light is in quantitative correlation with the number of viable tumor cells. We could

demonstrate that luciferase-transfected viable 4T1 cells emitting fluorescent light were significantly reduced already after two consecutive mEHT treatments.

This finding was confirmed by the histological analysis of hematoxylin- and eosin (H&E)-stained sections. The damage of the tumorous tissue was quantified on histological sections from the resected tumors by the Tumor Destruction Ratio (TDR), which was defined as the ratio of the whole tumor area and the damaged tumor area. On H&E-stained sections tissue damage appeared as pale, eosinophilic areas due to vacuolized cytoplasm and shrunk or fragmented nuclei of damaged tumor cells, which could be clearly differentiated from the morphologically undamaged cells. Following two mEHT treatments, TDR was significantly higher in the mEHT-treated tumors than in the sham-treated ones. We also demonstrated, that this significant tissue damage in the mEHT-treated tumors originating from the center of the tumors.

The damaged areas showed prominent signs of apoptosis and pronounced cleaved caspase-3 positivity, indicating that mEHT induced apoptotic cell death in the tumors.

We could show, that mEHT treatment induced significant hypocellularity in the tumors already after two treatments, which was accompanied by a significant reduction of tumor cell proliferation, when mEHT treatment was applied on a long term.

mEHT treatments provoked robust Hsp70 overexpression in the surviving tumor cells, implying the activation of the heat stress-related protective machinery of the tumor cells. However, we could show, that this tumor-protecting mechanism was exhausted by 48h after the last treatment. We could also demonstrate, that the significant tumor damage appeared by 24h after treatments, simultaneously with the exhaustion of the protective heat shock machinery. The treatment-induced tumor damage was pronounced and long lasting.

The effectivity of the mEHT treatments could be amplified significantly by combing mEHT with heat shock inhibitors – quercetin or KRIBB11 – *in vitro*, which abrogated the tumor protecting mechanism of the heat shock response. This finding has great translational potential.

4. Conclusions

The studies summarized in this thesis aimed to investigate the effects of modulated electro-hyperthermia in a triple-negative breast cancer animal model *in vitro* and *in vivo*. As a conclusion, our novel findings can demonstrate, that:

- a newly developed preclinical mEHT treatment device (LabEHY-200) could be optimized for treating orthotopic mammary tumors of small animals
- highly reproducible treatments could be performed with the optimized LabEHY-200 device *in vivo*
- highly effective and selective heating of the tumors could be achieved by mEHT treatments *in vivo*
- repeated mEHT treatments decreased the number of viable tumor cells *in vivo*
- repeated mEHT induced significant destruction of the tumors *in vivo*
- mEHT induced hypocellularity of the tumors already after two treatments *in vivo*
- mEHT inhibited proliferation by decreasing Ki67 expression after five treatments *in vivo*
- repeated mEHT induced caspase-dependent apoptosis *in vivo*
- repeated mEHT provoked Hsp70 overexpression *in vivo*
- the tumor destructive effect of mEHT is time-dependent *in vivo*
- the tumor cell killing effect of mEHT can be enhanced by combination with heat shock inhibitors *in vitro*

5. Bibliography of the candidate's publications

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