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Comparing the therapeutic potential of thermosensitive liposomes and hyperthermia in two distinct subtypes of breast cancer

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ABSTRACT

Local drug delivery of Doxorubicin (Dox) with thermosensitive liposomes (TSL) and hyperthermia (HT) has shown preclinically to achieve high local drug concentrations with good therapeutic efficacy. Currently, this is clinically studied for treatment of chest wall recurrence of breast cancer, however with various outcomes. This study examines the potency of neoadjuvant TSL HT combination therapy in two orthotopic mouse models of human breast cancer, MDA-MB-231 and T-47D, which morphologically correlate to mesenchymal and epithelial phenotypes, respectively. Both cell lines showed improved *in vitro* chemosensitivity and Dox uptake at HT. Dox-loaded TSL (TSL_{Dox}) was stable *in vitro* in FBS, BALB/c-nu plasma and human plasma, although release of the drug at HT was incomplete for the latter two. Combination treatment with TSL_{Dox} and HT *in vivo* was significantly more effective against MDA-MB-231 tumors, whereas T-47D tumors showed no significant therapeutic response. *Ex vivo* investigation revealed a higher mean vessel density and poorly differentiated extracellular matrix (ECM) in MDA-MB-231 tumors relative to T-47D tumors. Although *in vitro* results of the TSL_{Dox} and HT treatment were favorable for both cell types, the therapeutic efficacy *in vivo* was remarkably different. The well-differentiated and slowly-growing T-47D tumors may provide a microenvironment that limits drug delivery to the target cell and therefore renders the therapy ineffective. Mesenchymal and invasive MDA-MB-231 tumors display higher vascularization and less mature ECM, significantly enhancing tumor response to TSL_{Dox} and HT treatment. These results yield insight into the efficacy of TSL treatment within different tumor microenvironments, and further advance our understanding of factors that contribute to heterogeneous therapeutic outcomes in clinical trials.

1. Introduction

According to estimates made by the American Cancer Society, approximately 250,000 new patients are diagnosed each year in the US with invasive breast cancer and despite all medical progress, this disease will be responsible for about 40,000 deaths in the US in 2016 [1]. Chemotherapy is the standard of care for invasive breast cancer and can be applied as (neo)adjuvant therapy in addition to surgery or mastectomy for stage I-III tumors or for metastasized breast cancer (stage IV). Doxorubicin (Dox) is a drug that is used in many chemotherapy regimens as a single agent, in combination with other chemotherapeutic drugs, or as adjuvant for antibody-based therapies that target the estrogen receptor (ER), progesterone receptor (PR) or human epidermal growth factor receptor 2 (HER2). When a breast tumor lacks the expression of any of these three receptors, it is diagnosed as a triple negative breast cancer (TNBC). Depending on the stage of its diagnosis, TNBC can be particularly aggressive and is more likely to recur after local treatments compared to other subtypes of breast cancer. Lacking any response to receptor-targeted therapies, chemotherapy remains the only efficacious form of treatment [2]. However, severe side effects have been observed after treatment with Dox, including cardiac toxicity, nausea and hair loss, among others [3].

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These side effects can be largely reduced by incorporation of the drug in long-circulating nanoparticles such as liposomes. Due to their size of approximately 100 nm, the liposomes do not extravasate into healthy tissue, but passively accumulate in neoplastic tissue where the drug slowly diffuses out of its carrier. In fact, the use of liposomal Dox (Doxil®) has resulted in a similar therapeutic efficacy as free Dox, yet has greatly reduced the above-mentioned side effects [4,5], therefore becoming a preferred first-line single agent for stage IV TNBC [2]. However, further research has indicated that the parental drug Dox is actually retained too effectively inside the liposomes, which reduces its bioavailability and therefore strategies to exploit the full therapeutic potential of these drug delivery systems are warranted [6]. One solution is the development of thermosensitive liposomes (TSLs), which stably encapsulate the drug at body temperature and therefore reduce the side effects associated with free Dox, but enable fast Dox release when exposed to mild hyperthermia (40–42 °C; HT) [7]. This approach requires heating of the tumor area and has been shown in numerous preclinical studies to lead to higher accumulation of Dox compared to standard treatments, as well as improved tumor control [8–11]. The above-mentioned temperature-induced drug delivery may be particularly applicable for (neo)adjuvant treatment of local breast cancer or in case of local recurrence of breast cancer at the chest wall. Application to breast cancer has been performed in several subcutaneous murine models showing enhanced intratumoral Dox levels and therapeutic response [12,13]. Recently, a first clinical study was performed with a lysolipid-containing TSL (LTSL) formulation of Dox (ThermoDox®) in combination with HT for treatment of breast cancer recurrence at the chest wall showing a local response rate of 50% [14–16]. Though these results are promising, treatment response remains very heterogeneous and requires more investigation to further improve response rates.

Here, we present a study on the therapeutic efficacy of Dox-loaded, lysolipid-lacking TSLs for breast cancer. Removal of lysolipid from a thermosensitive formulation establishes a drug release by membrane defects in the liposome [17] instead of through pores established by the lysolipid (LTSL) [18]. Although our lysolipid-lacking TSL (hereafter abbreviated as “TSL”) has shown to slightly improve therapeutic outcome over LTSL [19], we focused in this study on tumor type comparison and relation to significant therapeutic response. For this comparison, ductal breast cancer orthotopic xenografts based on cell lines T-47D and MDA-MB-231 were selected. MDA-MB-231 is a TNBC, for which chemotherapy would be the standard of care, whereas T-47D expresses estrogen and progesterone growth receptors, rendering it susceptible to hormone therapy despite resistance remaining a problem in *e.g.* tamoxifen-based therapies [2,20]. T-47D cells belong to the luminal A class of breast cancer, which are well differentiated, epithelioid and relatively poorly invasive, therefore conferring a good prognosis [21]. MDA-MB-231 belongs to the basal/claudin-low class, is poorly differentiated and mesenchymal in nature, making it highly invasive, and resulting in a poor prognosis [22,23]. Investigating these extremes in breast cancer differentiation may provide new insights into the efficacy of preoperative TSL and HT based therapy among breast cancer subtypes.

2. Materials and methods

2.1. TSL preparation

1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC; Lipoid; Ludwigshafen, Germany) 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC; Lipoid), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-(amino(polyethylene glycol)-2000) (DSPE-PEG_{2000}; Lipoid) were dissolved in 9:1 (v:v) chloroform:methanol at a molar ratio of 70:25:5. The solvent was gradually evaporated by a rotary evaporator (Büchi; Flawil, Switzerland) and the resulting lipid was dried by nitrogen flushing. The lipid film was hydrated in $(NH_4)_2SO_4$ (250 mM, pH 5.3) to form liposomes which were extruded through 5 × 200 nm, 5 × 100 nm, 5 × 80 nm and 5 × 50 nm polycarbonate filters with a thermobarrel extruder (Northern Lipids; Burnaby, Canada). The liposomes were run over a PD-10 column (GE Healthcare Life Sciences; Eindhoven, Netherlands) and eluted with HEPES (10 mM) buffered NaCl (135 mM, pH 7.4). The phosphorus concentration of the sample was determined by ammonium molybdate spectrophotometry (Bartlett assay [24]) and liposomes were loaded with Dox (Actavis; Dublin, Ireland) by a $(NH_4)_2SO_4$ gradient as described before [25] at a 0.15:1 (mol:mol) Dox:lipid ratio, which was incubated for 1 h at 39 °C at 300 rpm in a thermoshaker. This $(NH_4)_2SO_4$ loading gave more stable TSLs at body temperature than the commonly used citrate loading [25], which has been used for LTSL [26]. Liposomes were concentrated by ultracentrifugation and the final product was tested for size, polydispersity and zeta-potential in HEPES buffer (10 mM) at a lipid concentration of 0.3 mM using a Zetasizer (Malvern Instruments; Worcestershire, UK).

2.2. In vitro Doxorubicin cytotoxicity assay

T-47D and MDA-MB-231 breast cancer cell lines were kindly provided by Dr. John Martens (Medical Oncology, Erasmus MC, Rotterdam, Netherlands). For chemosensitivity assays, cells were seeded in a 96-well plate and grown until 50% confluency, followed by addition of Dox and incubation at normothermic (37 °C; NT) or hyperthermic (42 °C; HT) temperature for 1 h. After incubation, the Dox-containing medium was removed and cells were washed with phosphate-buffered saline (PBS; Sigma-Aldrich; St Louis, Missouri). For investigation of the long-term effects of Dox incubation, fresh culture medium (RPMI medium + 10% FBS + 1% Penicillin/Streptomycin; Sigma-Aldrich) was added periodically. After a period of incubation (24–72 h), the cells were fixed in 10% (w:v) trichloroacetic acid (Sigma-Aldrich) for 4 h at 4 °C. Following fixation, the plates were washed with running tap water and cells were stained with 0.5% (w:v) sulforhodamine B (Sigma-Aldrich) for 20 min at room temperature. The plates were washed with 1% (v:v) acetic acid (Sigma-Aldrich) and left to dry, after which the stain was resuspended and homogenized by adding 10 mM Tris (Sigma-Aldrich). Absorbance was measured at 590 nm using a Wallac Victor 2 plate reader (Perkin Elmer; Waltham, Massachusetts). In case of a NT experiment, the Dox incubation on the cells took place in an incubator set to 37 °C, whereas a HT experiment was carried out by vacuum sealing the culture plate and submerging it into a water bath set to 42 °C. Cellular cytotoxicity and the following Dox uptake curves were only generated with free drug as previous studies showed that drug fully released from TSLs in culture media within seconds [27] and this study focused on an intravascular release approach in a heated tumor and thus bioavailable free drug to the tumor cell.

2.3. In vitro Doxorubicin uptake studies

Cells were grown until 80% confluency in a T75 culture flask and exposed to 40 μM Dox for 1 h, after which the cells were washed with ice-cold PBS. 40 mM was chosen as concentration Dox for an adequate fluorescent signal at the time of measurement. The cells were scraped from the flask in ice-cold PBS and centrifuged at 200 *g* at 4 °C for 10 min. 150 μL lysis buffer (20 mM Tris, 150 mM NaCl, 0.2% NP40, 10% glycerol, pH 7.4; Sigma-Aldrich) was used to resuspend the pellet and the resulting suspension was incubated on ice for 30 min. The lysates were centrifuged at 14,000 *g* for 15 min and pellets homogenized in 500 μL PBS by vortexing and 1 min probe sonication. Dox concentration was measured at 485 nm excitation and 580 nm emission by a Wallac Victor 2 plate reader. One additional T75 culture flask was seeded with cells in parallel to the flasks used for Dox uptake experiments in order to determine cell number at the time of the experiment.