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Targeted Thermosensitive Liposomes and Mild Hyperthermia for Local Drug Delivery to Tumors

Gerichte temperatuur-gevoelige liposomen en milde hyperthermie voor lokale afgifte van chemotherapeutica aan tumoren

THESIS

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List of abbreviations

CF	carboxyfluorescein
TEM	transmission electron microscopy
TSL	thermosensitive liposomes
CTSL	cationic thermosensitive liposomes
DMEM	Dulbecco modified eagle medium
Tm	phase transition temperature
SD	standard deviation
DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine
DSPC	1,2-distearoyl-sn-glycero-3- phosphatidylcholine
DSPE-PEG2000	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N PEG2000
PDI	polydisperisty index
GFP	green fluorescence protein
FCS	fetal calf serum
PBS	phosphate buffered saline
Em.	Emission
Ex.	Excitation
MRI	magnetic resonance imaging
HIFU	high intensity focused ultrasound
HT	hyperthermia
HPLC	high performance liquid chromatography
H&E	hematoxylin and eosin
LTSL	lysolipid-based thermosensitive liposomes
LLC	Lewis lung carcinoma
NT	normothermia
MSPC	monostearoylphosphatidylcholine
Dox	Doxorubicin
PEG	polyethyleneglycol
MPS	Mononuclear phagocytic system
EPR	Enhanced permeability and retention effect
AUC	Concentration-time curve
t ½	Plasma or circulation half-life
Vd	Volume of distribution
Cl	Clearance
RES	Reticuloendothelial system
PLD	Pegylated liposomal doxorubicin
IC50	Half maximal inhibitory concentration
NaCl	Sodium chloride
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
pdI	Polydispersity index
SEM	Standard error
DNA	Deoxyribinucleic acid

Contents

Chapter 1

Introduction

Cancer chemotherapy

Cancer chemotherapy is an important treatment option for primary (non-resectable) or metastatic tumors. Chemotherapy involves the use of cytotoxic drugs to destroy rapidly dividing cells such as cancer cells. Although an ideal drug would destroy cancer cells without harming normal cells, most drugs are not that selective. Since chemotherapeutic drugs target all cells having high proliferation rate such as hair follicles, intestinal cells and bone marrow cells, finding a unique target against which chemotherapy has selectivity is very difficult. Because chemotherapy is associated with side effects only sub-optimal doses can be administered, which can lead not only to relapse of the tumor, but also to development of drug resistance. The antracyclin doxorubicin has been in clinical use for several decades and is still among the most widely used chemotherapeutic drugs for treatment of cancer [1]. It is commonly used to treat different types of cancer, such as some leukemia's, Hodgkin's lymphoma as well as cancer of the bladder, breast, prostate, lung, ovaries, melanomas, carcinoma and sarcoma. Due to its intercalation with DNA to inhibit cell proliferation, doxorubicin can effectively kill cancer cells. However, Doxorubicin as a free drug has many side effects and its clinical use is hampered by cardiotoxicity, myelosuppression and the occurrence of drug resistance [2, 3]. The medical community has searched for alternative therapies that improve selective toxicities against cancer cells leading to an increase of the therapeutic indexes of the anticancer drugs [4]. To achieve this goal, nanoparticles such as liposomes were developed as a vector for therapeutic drug delivery in oncology and are supposed to break some of the physiologic and pharmacologic barriers to effective cancer drug treatment.

Liposomes

Liposomes are one of the best studied drug delivery systems used in cancer treatment. Nowadays several liposomal products are commercially available. Some of them are approved for clinical application and many more are in various stages of clinical trials [5]. Liposomes are closed vesicular structures consisting of one or more lipid bilayers enclosing an aqueous phase. In the aqueous phase of the liposomes water soluble drugs can be entrapped, whereas the lipid bilayer entraps lipid soluble drugs [6].

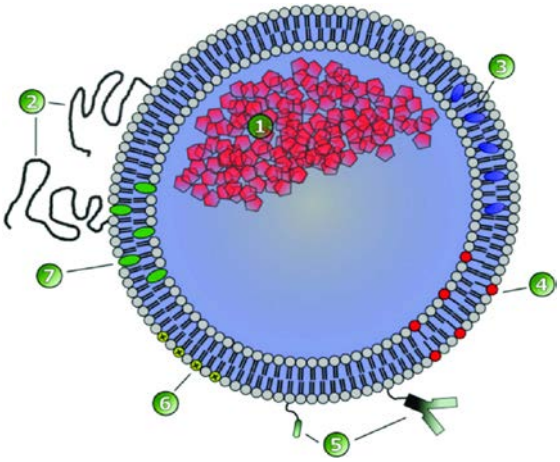

Fig 1. Schematic representation of a liposome. Loaded hydrophilic drugs into the aqueous phase (1). Polyethylene glycol coating (2). Lipophilic molecules embedded in liposomal bilayer (3). Lysolipids enabling drug release into liposomal bilayer (4). Surface modification with antibodies, antibody fragments or ligands for active targeting (5). Cationic lipids like DPTAP (6). Cholesterol for liposome stabilization (7). Figure is used from Hossann et al [7].

Amphiphilic drugs that are weak bases or weak acids can also be loaded into the liposome interior using remote loading methods like ammonium sulfate method for doxorubicin [8] or the pH gradient method for vincristine [9].

The use of liposomes has several advantages over the use of free drugs. They are non-toxic, biodegradable and non-immunogenic [10]. Another advantage of liposomes is that they have a long circulation time when equipped with a polyethyleneglycol (PEG) coating. These liposomes are also called Stealth liposomes [11]. Without this coating liposomes are rapidly cleared from the blood due to absorption of plasma proteins (opsonins) to the phospholipid membranes, triggering recognition and uptake of the liposomes by the reticuloendothelial system (RES).

Small 100 nm liposomes coated with PEG have half-lives of 12-30 hours in animal models and 21-54 hours in humans [12]. These circulation properties result in an increased accumulation of liposomal drug in the tumor area. This process of liposome accumulation is called passive targeting and it is due to the enhanced permeability and retention (EPR) of tumor tissue [13]. For efficient localization at the tumor site, the liposomes must have a diameter around 100 nm, which also depends on the tumor type.

A pegylated liposomal formulation of doxorubicin used clinically is called Doxil/Caellyx [14-16]. Doxorubicin is loaded into liposomes to a very high concentration by an ammonium sulfate gradient-based remote loading technique [8]. The loading efficiency is high (>90%). Loading is the result of the base exchange with the ammonium ions. Most of the intraliposomal doxorubicin is present in an aggregated state. The stability of the ammonium ion gradient is related to the low permeability of its counter ion, the sulfate, which stabilize anthracycline accumulation for prolonged

storage periods (>6 months) due to the aggregation and gelation of anthracycline sulfate salt [8]. Doxorubicin that is encapsulated in pegylated liposomes has a greatly altered pharmacokinetic profile, including the area under the plasma concentration-time curve (AUC), the half-life (t1/2), the volume of distribution (Vd) and the clearance (CL). The purpose of this alteration is to limit doxorubicin distribution into healthy tissue while increasing its concentration at the tumor site [10].

If pegylated liposomes are small enough they can pass through capillaries that have increased permeability, such as capillaries in the tumors [13, 17, 18]. This leakiness in the tumor vessels is due to basement membrane abnormalities and to decreased numbers of pericytes lining rapidly proliferating endothelial cells. The gaps size range from 100 to 1200 nm depending on the tumor type and even within the tumor [13, 19, 20]. This is in contradiction to the tight endothelial junctions of normal vessels of 5 to 10 nm size. Therefore, a proportion of tumor vessels is believed to be permeable and may allow extravasation of liposomes from them into interstitial space between the tumor cells.

Because doxorubicin is very slowly released from pegylated liposomes nearly all of the administered doxorubicin dose remains encapsulated in the carrier while in plasma [21, 22]. This together with the non-tumor specific nature of the liposomes leads to limiting steps in the delivery process and subsequently low therapeutic efficacy [16]. In this project we aim at developing new approaches to improve drug delivery into tumor cells. Liposomal drug delivery to tumors can be improved by using thermosensitive liposomes for triggering drug release upon hyperthermia (HT), by using cell-specific receptors for internalization into tumor cells or combining both targeted and triggered functions of liposomes in one carrier.

Hyperthermia

Mild hyperthermia (40-44 °C) has been used in the clinic as an adjuvant to radio- or chemotherapy and has been proven successful in treatment of various tumors-melanoma, sarcoma, head and neck cancer, breast cancer and cervix cancer [23-31]. The first large phase III clinical trial on combination of chemotherapy and HT has proven to be an effective treatment option for patients with localized soft-tissue sarcoma [24]. This effect of HT can be explained not only by causing cellular sensitization and immune effect but also by its influence on tumor microenvironment. HT is known to increase blood circulation, tissue oxygenation, tumor perfusion and vessel permeability [32, 33]. HT increases the gaps between endothelial cells causing extravasation of liposomes, thus increasing intratumoral liposomal drug accumulation [34-36]. Dewhirst [35] and Li [36] have shown that when tumors were heated to 42 or 41 °C respectively, liposomes extravasation increased as compared to tumors subjected to normothermia (NT). HT is also shown to trigger drug release from temperature-sensitive liposomes.

Thermosensitive liposomes

Another effect of hyperthermia besides the above mentioned is triggering drug release from thermosensitive liposomes (TSL). In this thesis, thermosensitive liposomes and HT are used as means to improve drug delivery to tumors. TSL consist of a thermosensitive phospholipid bilayer undergoing gel-to-liquid transition at its membrane melting temperature (Tm) [37]. The mobility of phospholipids is increased at Tm creating areas with increased permeability towards entrapped water soluble drugs [38]. In the past decade, TSL underwent several modifications in order to improve their stability [39, 40], blood circulation [41, 42] and release kinetics [42, 43]. Lindner et al. have used phosphatidyl-oligoglycerols in replacement of DSPE-PEG2000 for prolonged circulation and increased drug release at Tm [42]. Low temperature sensitive liposomes (LTSL) are used in intravascular release approach relying on fast drug release from liposomes subjected to HT while in circulation [44]. Kong et al. demonstrated in their study that doxorubicin concentration in the tumor was 3.6-fold higher when LTSL were used compared to traditional thermosensitive liposomes [45]. Recently, Al Jamal et al. compared the two formulations and showed that 1h after injection, LTSL delivered more doxorubicin to the tumor. However, 24h after injection, the amount of doxorubicin in the tumor was higher for the traditional thermosensitive liposome [46]. Tagami et al. developed similar LTSL using Brij surfactants replacing MSPC and DSPE-PEG2000. This formulation achieved faster drug release kinetics and higher doxorubicin levels in the tumor than LTSL [47-49]. Li et al. optimized the concentration of PEG in TSL giving an optimal content release upon mild HT [41] and showed that a thermal dose of 41 °C for 1h causes long-lasting permeable tumor vasculature to induce liposome extravasation and penetration into the tumor [36].

Targeted liposomes

Besides using an external trigger such as heat, increased drug delivery to tumors can be achieved by an active targeting of liposomes to tumors. Targeting of liposomes can be possible by decorating them with specific ligands recognizing receptors on tumor vessels or tumor cells. This specific recognition will yield increased liposome retention in tumors, liposome binding, liposome internalization into cells and subsequently increased drug delivery [10, 50]. Tumor vascular targeting has a great role in suppressing tumor development as blocking tumor vasculature growth will decrease nutrients and oxygen to tumor cells [51]. Amongst the most specific targets on endothelial cell membranes are αvβ3 integrins [52], receptors for angiogenic growth factors (such as VEGFR, EGFR) [53], aminopeptidase N (e.g CD13) [54] and the overexpression of negatively charged molecules [55]. Tumor vascular targeting is a promising way of killing tumors as tumor endothelial cells are easy to access and high interstitial fluid pressure does not play a role. Anti-VEGFR2 immunoliposomes developed by Wicki et al. [53], NGR-targeted [54] and RGD-targeted liposomes [52, 56, 57] have proven successful in diminishing tumor progression. Additionally, overexpressed anionic molecules on endothelial cell membranes can be target for cationic liposomes based on electrostatic interaction [58, 59]. Various studies have shown that cationic liposomes were able to inhibit

tumor growth when compared to non-cationic formulations [60, 61]. Besides vasculature targeting, taking the advantage of their small size and prolonged blood circulation, liposomes can extravasate and reach tumor cells. Once in close proximity with tumor cells, liposomes can bind and be internalized into tumor cells due to receptor-mediated endocytosis. Liposomes decorated with antibodies or peptides have been developed to successfully target tumor cells. For example, liposomes anchored with HER2 antibody are used to target HER2 overexpressing breast cancer cells [62, 63]. EGF receptor overexpressed on tumor cells is another potent receptor for targeted liposomes and these liposomes have demonstrated higher tumor suppression when compared to non-targeted liposomes [64]. Targeted liposomes bring the drug close to tumor vasculature or cells but the drug is usually released very slowly which hampers its bioavailability. In order to improve drug bioavailability, targeted thermosensitive liposomes appear as promising drug carriers in the fight against cancer.

Targeted thermosensitive liposomes

Combining targeting and thermosensitive characteristics of liposomes in creation of targeted thermosensitive liposomes (TTSL) can contribute to enhanced drug bioavailability by increasing liposomal drug retention in tumors, binding and triggering drug release locally in the tumor. TTSL may be developed by attaching to their outer surface specific peptides or antibodies or incorporating cationic lipids in their bilayer.

The first TTSL were developed by Gaber et al. and were targeted to a folate receptor on tumor cells [65]. The recently developed affibody-conjugated HER2 thermosensitive liposomes were 10-fold more specific in HER2 positive tumor cells than non-targeted liposomes [66, 67]. The studies by Kullberg et al. have shown that the incorporation of lysteriolysin 0 in the liposomes can form pores in the endosomal membrane and thus enable transition of released liposomal drug into the cytosol. It also showed that TTSL to HER2 positive cells delivered 22-fold higher amount of liposomal calcein to HER2 positive cells compared to HER2 negative [68, 69]. Dreher et al. [70] has created TTSL to tumor vasculature by using NGR peptides on the liposomal surface. This emerging field in liposomal drug delivery shows promising results but still more in vivo work is needed in order to prove the efficiency of TTSL.

AIM OF THE THESIS

The aim of the work described in this thesis is to improve liposomal drug delivery to solid tumors by using the combination of targeted thermosensitive liposomes and mild HT. The designed liposomes contained targeting moieties specific not only for tumor cells but also for tumor vascular endothelial cells. Targeting of liposomes is used to increase their retention in tumors, therefore contributing to increased drug delivery. In this thesis, two different targeted thermosensitive liposomal formulations are used - cationic TSL specific for overexpressed anionic molecules on tumor vascular endothelial cells and tumor cells; and RGD-TSL having affinity for αvβ3 integrins on

tumor vasculature and tumor cells. Local mild HT is used to increase permeability in tumor vasculature for liposomal extravasation and to enhance fluid flow thus enabling liposomal drug penetration in the tumor. When a maximum liposome accumulation occurs, a second heat treatment is applied to trigger drug release from liposomes. Liposomes with melting temperature at Tm are used as drug carriers to achieve triggered drug release. Choosing the best concentration of targeting ligands in order to achieve maximum targeting and drug loading efficiency are a matter of this thesis.

Topics of the thesis

Chapter 2 describes the development of cationic thermosensitive liposomes (CTSL) containing the hydrophilic marker carboxyfluorescein (CF) in the aqueous phase. CTSL were tested in vitro for stability at physiological temperatures and their drug release kinetics were investigated. In vitro, CTSL were compared to non-cationic thermosensitive liposomes (NCTSL) regarding their binding capacities to tumor cells and endothelial cells. In vivo, using advanced intravital microscopy techniques, we investigated the stability, binding and drug release of CTSL.

Chapter 3 focuses on optimization of CTSL containing the chemotherapeutic drug doxorubicin with regards to their stability in serum, drug encapsulation and targeting functions. CTSL containing 7.5 mol % of the cationic lipid DPTAP was chosen for further experiments. The optimized Dox-CTSL are compared in vitro and in vivo to NCTSL loaded with doxorubicin with regards to cytotoxicity, drug release and uptake by endothelial and tumor cells and subsequently caused vessel damage.

Chapter 4 studies the design of RGD-targeted thermosensitive liposomes incorporating doxorubicin. These liposomes were compared to their non-targeted formulation in vitro and in vivo aiming at increased drug delivery to tumors.

Chapter 5 describes pharmacokinetics, biodistribution and therapeutic effect of TSL and CTSL.

Chapter 6 reviews the literature on TSL, targeted liposomes and their combination in the creation of targeted thermosensitive liposome (TTSL), which are the topic of this thesis.

Chapter 7 discusses the results of the different chapters.

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Chapter 2

Cationic thermosensitive liposomes - a novel dual targeted heat-triggered drug delivery approach for endothelial and tumor cells

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Abstract
Developing selectively targeted and heat-responsive nanocarriers holds paramount promises in chemotherapy. We showed that this can be achieved by designing liposomes combining cationic charged and thermosensitive lipids in the bilayer. We demonstrated, using flow cytometry, life cell imaging and intravital optical imaging, that cationic thermosensitive liposomes (CTSL) specifically target angiogenic endothelial and tumor cells. Application of mild hyperthermia led to a rapid content release extra-and intracellularly in two crucial cell types in a solid tumor.

Keywords: cationic thermosensitive liposomes, hyperthermia, triggered release, drug delivery, targeted nanomedicine

Nanoparticles, such as liposomes can preferentially deliver chemotherapeutics to tumors due to their small size and long circulation half-life in combination with the leakiness of tumor blood vessels.[1,2,3] The increased permeability of tumor blood vessels is due to angiogenesis related endothelial cell proliferation, basement membrane abnormalities and irregular perycite lining. In contrast to normal vascular endothelium, which is continuous, tumor endothelial gap sizes can range from 100 to 400 nm.[4] In addition to increased permeability, tumor blood vessels have a chaotic architecture and irregular branching.[5] Small liposomal nanoparticles of around 100 nm were proven optimal for extravasation at sites with increased permeability.[6] This in combination with decreased clearance and enhanced blood-residence time of the liposome-entrapped drug, will decrease drug exposure to normal tissue and increase drug concentrations in the tumor.[7] In spite of the advantage of reduced toxicity to normal tissue demonstrated by clinically applied liposomal chemotherapy, a significant enhancement of therapeutic efficacy was not observed.[8,9] The two main reasons for this are the low levels of retention of the liposomes in the tumor area as well as the slow and inefficient drug release from liposomes. The aim of this study is to develop a liposomal formulation which deals with both drawbacks and thereby improves intratumoral drug levels. In order to achieve improved retention we aim for cell-specific targeting and carrier internalization by introducing cationic charges on the particle surface. In addition, a thermally sensitive bilayer is used to render liposomal drug delivery heat responsive.

Campbell et al.[10] demonstrated that cationic liposomes have higher affinity for angiogenic endothelial cells and tumor cells, compared to normal tissue, while PEG coating ensured long circulation time. The increased binding of PEGylated cationic liposomes to tumor vessels is due to the overexpression of negatively charged functional groups on the angiogenic endothelial cell membrane.[11,12] Moreover, the sluggish and irregular blood flow in tumor blood vessels further facilitates the interaction between cationic liposomes and angiogenic endothelial cells.[10] Additionally, Bally et al.[13] have shown that cationic liposomes might be opsonized by a yet unidentified plasma protein, causing specific binding of these liposomes to angiogenic endothelial cells. Drugs

delivered to tumor endothelial cells in this way can induce damage to tumor microvasculature and eventual loss of tumor vessel function. As a result, a large number of tumor cells may die because of severe oxygen and nutrient deprivation.[14] In addition, cationic PEG-liposomes may also bind to tumor cells upon extravasation through leaky tumor vasculature[15,18,19], a process which can be further enhanced by applying hyperthermia (HT).[16, 17]

Despite localization of liposomal drugs in tumors, drug bioavailability is not guaranteed. Seynhaeve et al. observed in vivo that after extravasation of clinically applied PEG-liposomal doxorubicin (Doxil/Caelyx) into the interstitial space, intracellular drug delivery is rather slow and inefficient, resulting in suboptimal bioavailable drug concentrations.[18] Mild HT (40 - 43 °C) can trigger drug release locally from thermosensitive liposomes (TSL).[20,21] Thermally sensitive bilayers can be obtained by selecting lipid compositions that undergo a gel-to-liquid phase transition at the desired temperature, causing release of entrapped water soluble compounds. TSL deposit their contents in heated tumors in the extracellular space after extravasation or intravascularly when still in circulation. However, TSL retention in tumors is low due to PEG coating and a lack of specific targeting, limiting drug delivery to the tumor. In order to improve this liposomal chemotherapy we propose to combine cationic charge mediated targeting with thermally triggered content release.

This study reports on the development and application of PEGylated cationic thermosensitive liposomes (CTSL). These liposomes were used in combination with hyperthermia for a dual-targeting approach to address both angiogenic endothelial cells and tumor cells. After targeting, these nanoparticles can be triggered to release their contents upon heat. This novel approach combines for the first time the dual targeting by cationic lipids with temperature-triggered release and holds promise to improve chemotherapeutic drug delivery.

CTSL and NCTSL (noncationic thermosensitive liposomes) were composed of the same phospholipids DPPC: DSPC: DSPE-PEG_{2000}. In addition to the phospholipids, CTSL contained 10 mol % of the cationic lipid DPTAP in their bilayer in replacement of a similar amount of DPPC. Both formulations were prepared by the lipid film hydration and extrusion method. [22] Prepared nanoparticles were characterized by measuring size, polydispersity index (PDI), zeta potential (ζ), stability in serum, T_m (melting temperature) and encapsulation ratio (table 1). Sizes of NCTSL and CTSL were slightly less than 100 nm and both TSL had a polydispersity index (PDI) < 0.1, representing a high level of particle homogeneity. CTSL had a positive zetapotential, whereas NCTSL displayed a negative zeta potential, which is common for PEG-DSPE containing liposomes due to its shielded negatively charged phosphate group. NCTSL encapsulated two times more carboxyfluorescein (CF) than CTSL and both formulations retained > 90% of their contents at physiological conditions, during incubation for 1h at 37 °C in 90% of serum. Dynamic scanning calorimetry (DSC) revealed for NCTSL a relatively narrow peak in the phase diagram with a T_m of 44,3 °C and $\delta T1/2$ (half-height width of the transition peak) of 3,1 °C. For CTSL a broader peak was observed

with a T_m of 47,4 °C and δT1/2 of 7,3 °C. The onset in the transition diagram was at approximately 40 °C for both formulations (see Supporting information fig1).

Table 1. Characterization of NCTSL and CTSL. Mean of three independent experiments. PDI: polydispersity index.

Batch	Size (nm)	PDI	Zeta potential (mV)	Encapsulation ratio (mole/mole of CF/lipid)	Stability in 90% FCS at 37 °C in 1h	T_m (°C)
NCTSL	84 ± 3	0.075 ± 0.01	-11 ± 0.7	0.10 ± 0.020	99.8 ± 0.4	44,3
CTSL	91 ± 2	0.070 ± 0.01	13 ± 1.0	0.05 ± 0.009	92.0 ± 3.0	47,4

In order to test thermosensitivity of the developed CTSL and kinetics of content release upon hyperthermia, temperature- and time-dependent release profiles were determined. CTSL displayed a clear temperature dependent CF release profile upon exposure for 5 min in 90% FCS to temperatures varying between 37 and 45 °C (fig 1A). CF release from CTSL increased with increasing temperature up to 45 °C, reaching 80% release in 5 minutes. Between 40 and 43 °C, release from CTSL and NCTSL were virtually identical. However, maximum CF release from NCTSL of 60% was observed at 43 °C, after which release levels decreased at higher temperatures. By contrast, release from CTSL continued to increase between 43 and 45 °C, which is in agreement with the increased T_m for CTSL represented in Table 1. Importantly, CF release from both formulations remained low (< 15%) at physiological temperatures of up to 39°C.

Rapid content release from CTSL at 42 °C was demonstrated during a 1h time-dependent CF release assay in 99.7% FCS (fig 1B). CTSL released up to 50% of their content in the first minute, similar to NCTSL. CF release continued to increase in time reaching approximately 100% release of entrapped CF in 1h from both formulations. By contrast, using similar conditions, but now at a temperature of 37 °C, hardly caused any content release from CTSL (Table 1).

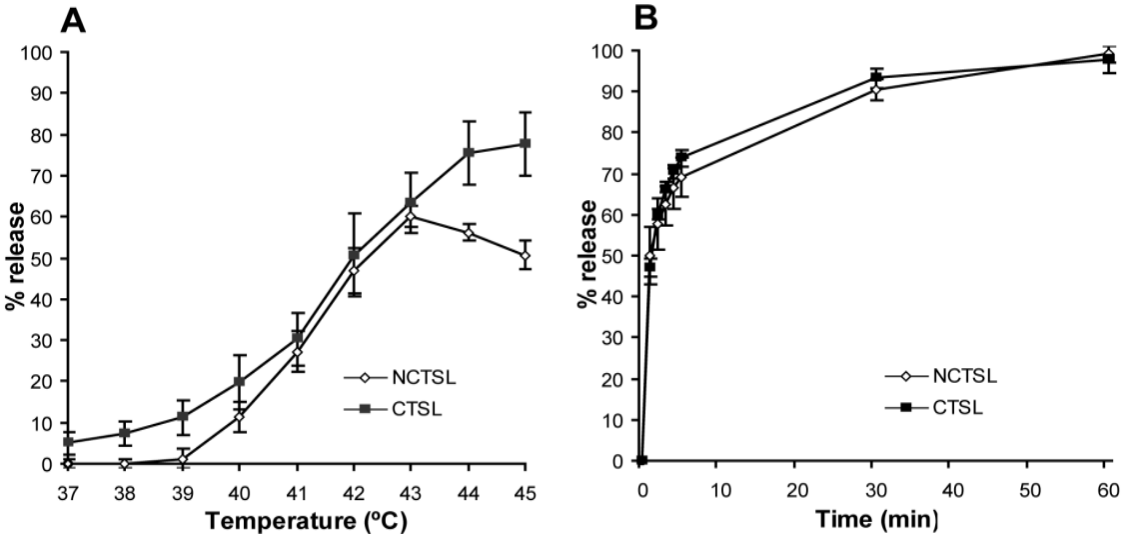

Fig 1. Temperature- and time-dependent CF release from CTSL and NCTSL. Temperature-dependent release of CF (A) was measured upon exposure to temperatures varying from 37 - 45 °C for 5 min in 90% FCS. Whereas NCTSL displayed maximum release around 43 °C, CTSL showed increasing CF release up to a maximum level at 45 °C. Time-dependant CF release (B) was measured at constant temperature of 42 °C for up to 1h in 99.7% serum. Both TSL displayed rapid CF release in the first five minutes and were emptied completely in 1h. Mean of three independent experiments with three independent batches of liposomes.

To compare binding of CTSL and NCTSL to BLM tumor cells and HUVEC, flow cytometric cell association studies were performed (fig 2). For these studies, trace amounts of the fluorescently labeled phospholipid, NBD-PE, were incorporated in the TSL bilayer. CTSL showed up to 50-fold higher level of association with BLM cells at 37 °C (fig 2A) and up to 25-fold increase at 4 °C (see Supporting information fig 2A). The same tendency for increased binding of CTSL was observed in HUVEC as it was up to 7-fold higher than NCTSL at 37 °C (fig 2B) and 4-fold higher at 4 °C (see Supporting information fig 2B). Levels of binding of CTSL to both BLM cells and HUVEC were higher at 37 °C than at 4 °C. These differences are suggestive of CTSL internalization occurring at 37 °C.

In order to further prove CTSL binding and internalization, living cell confocal microscopy was performed (fig 2C and D). Incubation for 1h at 37 °C led to abundant internalization of CTSL by BLM cells (fig 2C) and HUVEC (fig 2D), compared to NCTSL. Liposomes were observed in the cytosol of both BLM and HUVEC in punctuate red fluorescent spots of Rho-PE (rhodamine-PE) located perinuclearly, proving internalization. Fluorescent CTSL incubated for 1h at 4°C with BLM showed a high level of membrane bound fluorescence, in contrast to NCTSL (see Supporting information fig 2C). A similar experiment with HUVEC revealed minimal differences

between the two formulations at 4° C, and confirm the FACS results on liposome binding (see Supporting information fig 2B).

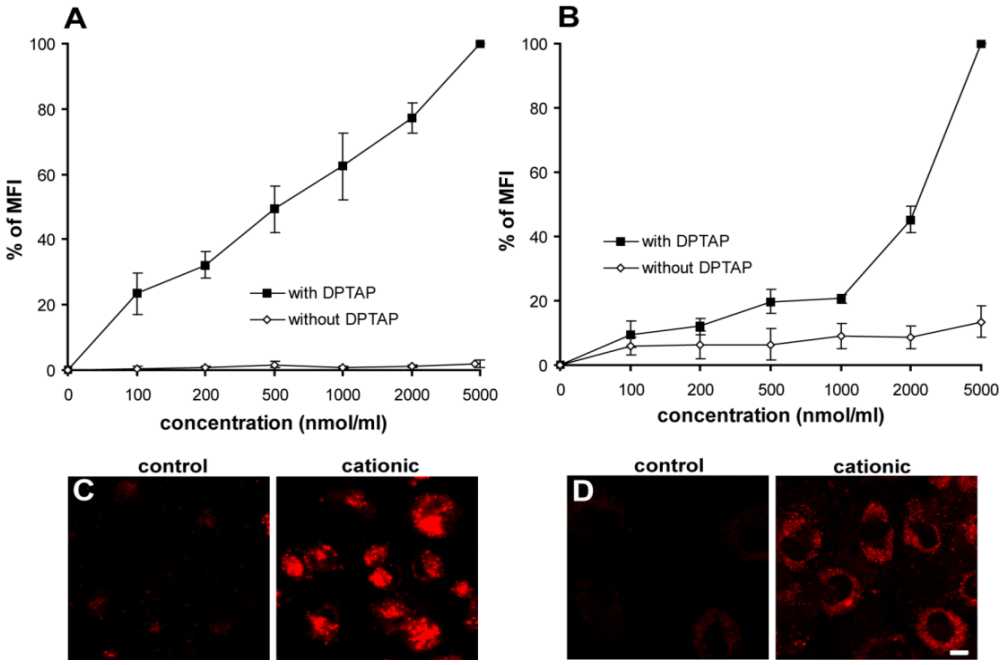

Fig 2. Binding of CTSL or NCTSL to BLM melanoma cells (A) and endothelial cells (HUVEC) (B) measured by flow cytometry and confocal microscopy images proving binding and internalization of CTSL or NCTSL by BLM cells (C) and HUVEC (D). A and B. Cells in suspension were incubated for 1h with NBD-PE labeled CTSL or NCTSL at 37 °C. After washing, the cell-associated NBD-PE fluorescence was determined by flow cytometry. Values depicted in the figure represent the mean fluorescent intensity calculated as a percentage of the maximum mean fluorescent intensity from three experiments with three different batches of liposomes. C and D. BLM or HUVEC cells were incubated with Rho-PE labeled liposomes for 1h at 37 °C. After incubation, cells were washed and visualized by confocal microscopy. Scale bar apply for all images, 10 µm.

Upon binding and subsequent internalization of CTSL, release of content from the nanoparticles can be triggered by increasing temperature and may occur both extra- and intracellularly.

Extracellular content release was studied by incubating BLM cells at 4 °C for 1h with CF-loaded CTSL, which had Rho-PE as a membrane marker, followed by a hyperthermia treatment of 1h at 43 °C. CF dequenching upon release from the CTSL was visualized by its green fluorescence and used as a marker for content release. After the binding phase and removal of unbound liposomes, hyperthermia at 43 °C caused a rapid content release from the cell-bound CTSL represented by a strong increase in

green fluorescence in the medium surrounding the cells in contrast to cells that were kept at 37 °C (Fig 3A and see Supporting information video 1).

During the binding phase (1h at 4 °C) to BLM cells and the heating phase up to 37 °C, no premature extracelular CF leakage was observed. Only upon HT, liposomes opened up and started to release their CF content extracellularly. Extracellular CF fluorescence continued to increase in time up to 1h of hyperthermia. In this experimental setup occasionally intracellular content release was observed (fig 3A white arrows) from liposomes internalized after raising the temperature.

Intracellular content release in BLM cells was demonstrated by incubating the cells at 37 °C for 1h, allowing internalization, followed by a 1h heating at 43 °C (fig 3B). Minimal CF release was observed during the incubation at 37 °C, whereas HT caused a strong increase in intracellular CF release as demonstrated by the increase in yellow fluorescence which represents colocalization of hyperthermia-released CF with the liposomes in the cytosol (see Supporting information video 2).

The experimental set up was adapted for HUVEC cells to a shorter incubation time due to their fast uptake and processing of CTSL compared to BLM cells. Extracellular and intracellular content release from CTSL were observed simultaneously in HUVEC incubated for 15 min at 4 °C, followed by removal of unbound liposomes, heating to 37 °C and a 1h HT at 43 °C (Fig 3C). Images taken at 37 °C before heating showed minimal CF release. Increasing the temperature to 43 °C caused immediate CF release, which increased gradually during the 1h heating period. In addition to extracellular release, significant intracellular release was also visible in HUVEC from the appearing green fluorescent hotspots with increasing intensity inside the cells or the increased yellow fluorescent signal inside the cells representing colocalization of the released CF with the liposomes (Fig 3C indicated by white arrows).

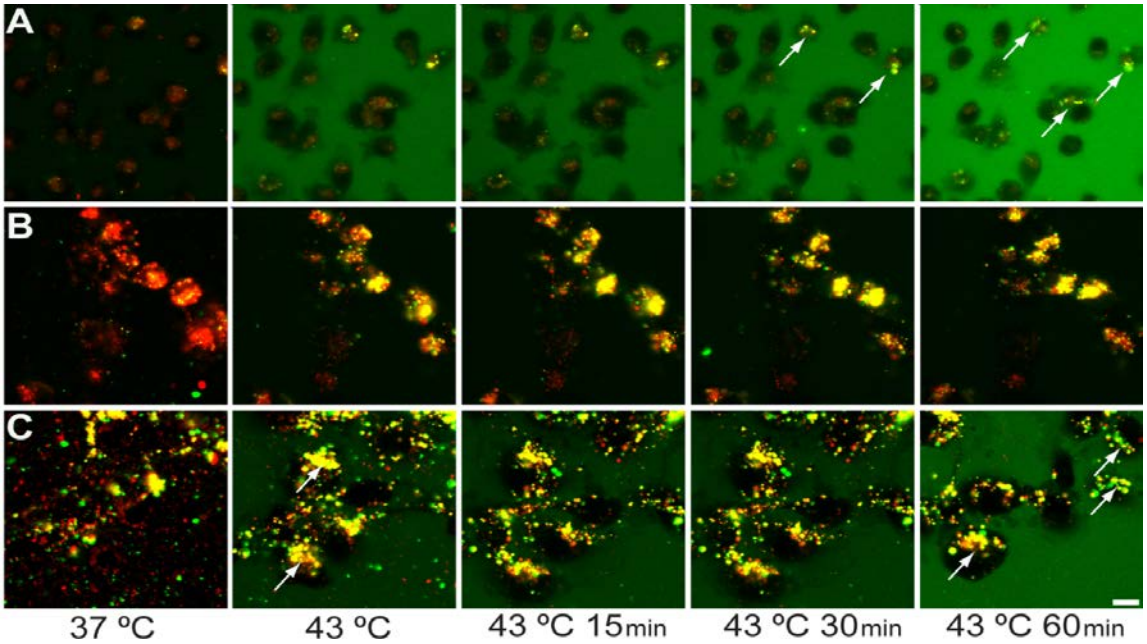

Fig 3. Content release from CTSL in BLM cells and HUVEC upon heat. Extracellular CF release in BLM cells (A) incubated for 1h at 4 °C with 1000 nmol/ml dual labeled Rho-PE CTSL (red color), encapsulating CF (green color) and intracellular release in BLM cells (B) incubated for 1h at 37 °C with 1000 nmol/ml dual labeled CTSL. In both conditions content release was triggered by a one hour hyperthermia treatment at 43 °C. Extra- and intracellular release in HUVEC (C) pre-incubated for 15 min at 4 °C with 1000 nmol/ml Rho-PE CTSL, containing CF followed by HT at 43 °C. After CTSL incubation, cells were washed 3 times with medium containing FCS. Images were taken by confocal microscope. Scale bar apply for all images, 10 µm.

Further proof of the validity of the dual targeting approach to angiogenic endothelial cells and tumor cells in vivo, was obtained by intravital microscopy. Dorsal skin-fold window chamber bearing mice were implanted with murine B16 melanoma or murine LLC lung carcinoma.[15] In these mice tumor vasculature is visualized by the constitutive expression of a GFP-Enos-tag fusion protein in endothelial cells. Circulating liposomes in the blood stream were visualized by incorporation of Rho-PE in the bilayer of TSL (red). Fluorescence from labeled CTSL was diffusely distributed in the lumen of blood vessels in the first 15 minutes after injection. At later time points CTSL started to appear closely associated with the endothelial cells predominantly of blood vessels with a slow and irregular blood flow in both B16 (fig 4A,B,C,D) and LLC tumors (see Supporting information fig 3A), as represented by the appearance of patchy clusters of high intensity immobile red fluorescence in the blood vessels. This is in contrast to the NCTSL, which remained diffusely distributed without obvious binding to the vasculature even in tumor vessels with irregular blood flow (fig 4F and Supporting information fig 3B). Images in B16 melanoma taken 24h after injection of CTSL demonstrated considerable levels of remaining liposomes either associated with the tumor vasculature of functional blood vessels or extravasated from the vasculature and accumulated perivascularly (fig 4E white arrows).

In order to prove direct binding of CTSL to angiogenic endothelial cells, we excluded possible binding of CTSL to circulating blood cells. Blood cells isolated from mice 2h after i.v. administration of Rho-PE labeled CTSL encapsulating CF did not show associated fluorescence (see Supporting information fig 4). Therefore, the specific binding of CTSL to angiogenic endothelial cells or the extravasation of the liposomes did not involve prior binding or interaction with circulating cells.

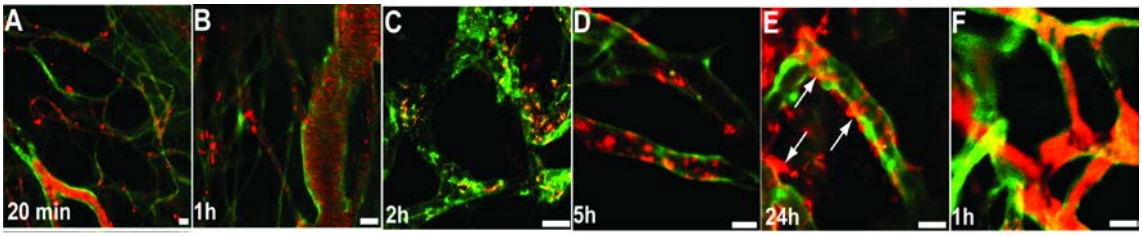

Fig 4. Intravital evaluation of binding of CTSL (A, B, C, D and E) or NCTSL (F) to angiogenic endothelial cells in B16 tumor model using the dorsal skin-fold chamber. Mice were injected with 5 µmol of lipid. Appearance of vasculature bound cationic liposomes started 20 min after injection and remained visible at least up to 24h. At that time point CTSL were found associated with tumor vasculature or extravasated perivascularly (E white arrows). NCTSL hardly bound to the endothelial lining of tumor associated blood vessels (F). Scale bar apply for all images, 20 µm.

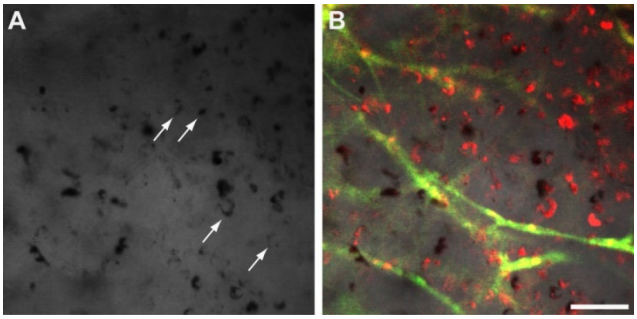

Fig 5. Binding of CTSL to melanoma cells in B16BL6 tumor model extravasated from tumor vessels (green) 3h after injection of CTSL. CTSL (in red) associated with melanoma cells (black, melanin-positive) are pointed with white arrows. A-bright field image, B-merged image of bright field, green and red fluorescence. Scale bar 50 µm.

Upon arrival in the tumor tissue CTSL should release their content upon heat. Therefore, triggered content release from CTSL was studied by intravital microscopy. After CTSL had been allowed to bind to tumor vasculature and tumor cells during a period of 3h at 37 °C, the effect of HT on CF release in vivo was examined. For this purpose, images of different positions in the tumor area were first recorded at 37 °C (fig 6 upper panel, tumor vasculature lining is represented in green from GFP-expressing endothelial cells). At this temperature no CF release (additional green fluorescence) could be detected in the tissue or vasculature, yet CF-containing liposomes were still in circulation as is apparent from the red fluorescence in the tumor microvessels. Upon applying HT at 43 °C, a massive CF release occurred (fig 6 lower panel). HT was applied for 1h and images taken at the end of the HT treatment were compared to those taken at 37 °C. Strong increases in the green fluorescence demonstrate massive heat triggered CF release from CTSL followed by its diffusion into the tumor tissue. The CF

fluorescence increased by 3-fold upon HT (see Supporting information fig 5A). HT also led to further increase of the binding and the extravasation of CTSL as evidenced by the increased level of yellow fluorescence appearing in patchy patterns on the tumor blood vessels and extravascularly. Rhodamine fluorescence increased 2-fold after 1 h of HT (see Supporting information fig 5B). All together, these results demonstrate HT-triggered content release from CTSL 3h after administration and a further increased tumor retention of CTSL.

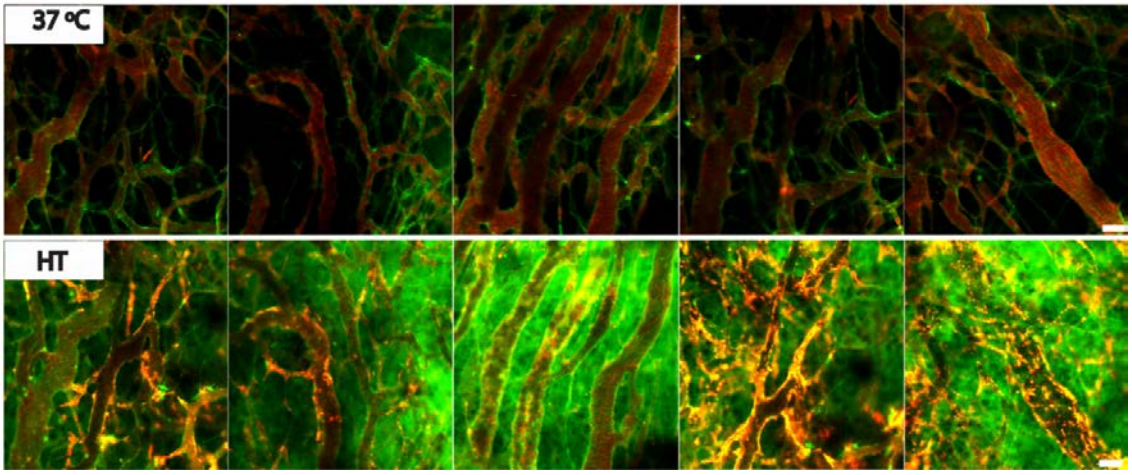

Fig 6. CF release after HT triggering in B16 tumor bearing mouse, injected with 5 µmol of lipid. Images of circulating liposomes in blood stream at 37 °C were taken 2h after injection. Then, tumor was heated up to 43 °C for 1h and representative images from different positions in tumor area were recorded in the end of the hyperthermia (HT) treatment. Scale bar apply for all images, 50 µm.

Liposomal chemotherapy has become an attractive clinical option in the treatment of various types of cancer, mainly because of their capacity to diminish side effects. [1, 8, 30, 31] However, the anticipated increase in therapeutic efficacy did not yet come to its full potential.[8,9] The reasons for this are the limited tumor accumulation, drug bioavailability and lack of control of content release. In this study we addressed these issues by developing tumor targeted thermosensitive liposomes. For this we combined the approach of cationic charge mediated targeting of PEG-liposomes with heat triggered content release and successfully developed cationic thermosensitive liposomes (CTSL). These CTSL demonstrated content release kinetics similar to NCTSL that were proven successful for in vitro and in vivo heat triggered content release recently [21], i.e. they were stable at physiological temperatures of 37 °C and showed rapid content release at 41 °C or higher, temperatures which can be applied in clinical mild hyperthermia (41-43 °C). In addition to the triggered release, CTSL showed increased binding to endothelial and tumor cells in vitro and in vivo. Upon binding, CTSL were internalized and their release could, in addition to extracellularly,

also be triggered intracellularly. Most importantly, in vivo we were able to prove the dual targeting strategy by demonstrating binding of CTSL to tumor vasculature in combination with extravasation and subsequent tumor cell binding, and temperature-triggered content release only upon heat.

CTSL displayed high stability (< 10% release of their content) upon exposure to physiological conditions during 1h of incubation at 37 °C in 90% serum. The temperature dependent release assay showed that initial CF release mainly occurred in the first 5 min after which the liposomes apparently are stable. This small fraction released, may well represent anionic CF that has been electrostatically associated with DPTAP at the liposome surface and is released upon exposure to the biological environment with 90% of serum.

The release kinetics of both formulations are consistent with their T_m. NCTSL have a T_m of 44,3 °C and show maximal release before reaching this temperature. Most likely NCTSL membranes that reach a homogeneous liquid crystalline phase at their T_m have less permeability at higher temperatures. Apparently, at temperatures just under their T_m maximal bilayer defects occur, allowing maximal CF release. CTSL displayed a higher T_m of 47,4 °C and a broader phase diagram, which explains the observation that release continued to increase up to 45 °C. The higher T_m of CTSL can be explained by the addition of DPTAP in the bilayer, which has a T_m of 49,3 °C. In addition, cationic lipids are known to cause an increased T_m of phospholipids [23], an effect which can be explained by the electrostatic interactions between the cationic DPTAP and the zwitterionic phospholipids, which changes the thermotropic behavior of the latter.[24] Importantly, in good correlation with the onset temperature of the phase diagram, significant content release from CTSL already occurred between 40 °C and 43 °C, temperatures that are feasible in clinical hyperthermia.

Additionally, CTSL have a positive zeta potential (table 1), which increased their binding to tumor and endothelial cells compared to NCTSL. The level of cell association of CTSL to both BLM and HUVEC cells was higher at 37 °C than at 4 °C, a temperature at which internalization processes are blocked, indicating that at 37 °C liposomes are internalized after cell surface binding, a finding that was confirmed by confocal laser scanning microscopy.

Besides CTSL binding and internalization, we also demonstrated HT-triggered liposomal content release both in vitro and in vivo. In vitro, using living cell microscopy we showed both extra- and intracellular CF release upon applying HT on BLM and HUVEC cells.

In consistence with the in vitro results, the results from intravital microscopy also demonstrate a preferential binding of CTSL to tumor vascular endothelial cells. In accordance with Thurston et al. who studied non thermosensitive cationic nanoparticles [25], our CTSL associated with endothelial cells already 20 min after injection and showed a patchy appearance at multiple locations lasting for several hours in both B16 and LLC tumor models (fig 4A,B,C,D,E and Supporting information fig 3A). In contrast, NCTSL remained in circulation and did not bind to the vasculature and appeared diffusely present in the vessel lumen (fig 4F and Supporting information fig 3B). In accordance

with Krasnici et al.[26] who showed long term presence of cationic liposome in tumors up to 6h after administration, we were able to demonstrate long-term retention of CTSL, associated with tumor blood vessels, up to 24h after injection. Additionally, our CTSL did not show binding to blood cells, which indicates that their specific binding is most likely directly to angiogenic endothelial cells. Binding of CTSL to tumor vasculature most likely involves similar mechanisms as described by Campbell et al.[10] showing that the sluggish and irregular blood flow in combination with increased anionic charges on the endothelial cell membranes enhanced binding of cationic liposomes to angiogenic endothelial cells. Binding events that we observed with CTSL were multiple in tumors and usually in areas with slow and chaotic blood flow. Remarkably, HT strongly enhanced binding of CTSL to tumor vessels and promoted further extravasaton and therefore could, besides triggering content release, also further enhance tumor retention of these nanoparticles.[27, 6] This effect can be related to an increase in the exposure of negative charges on endothelial cell membranes as a response to the HT, leading to increased binding of CTSL.

Besides binding to the tumor vasculature, our CTSL, due to their small size and prolonged presence in circulation, extravasated from the leaky tumor vasculature and bound to tumor cells, thus achieving dual targeting properties.

In vitro stability of our CTSL at 37 °C was also confirmed in vivo. Intravital optical imaging at body temperature displayed minimal CF release over a period of 2h before HT was applied. HT at 43 °C 2 hours after liposome injection however, caused massive CF release, which was observed in numerous positions throughout the tumor area (fig 6). In our in vitro and in vivo studies we did not observe (cellular) toxicity of our cationic liposomes, which is in line with earlier findings.[28,29] Future studies, in which CTSL will be loaded with chemotherapeutic drugs will address biocompatibility of our novel nanoparticles in more detail.

In conclusion, thermosensitive cationic liposomes have been developed that are stable at physiological temperatures and release their contents extra or intracellularly upon application of mild hyperthermia both in vitro and in vivo. These new CTSL bind to tumor vascular endothelial and tumor cells both in vitro and in vivo. PEGylated CTSL in combination with mild HT can increase drug delivery to tumors because of their selective targeting properties together with heat triggered content release. With the use of CTSL, a dual targeting and internalization strategy for both tumor vascular endothelial cells and tumor cells can thus be achieved. Further studies will focus on developing CTSL, encapsulating a chemotherapeutic drug for targeting tumor vasculature, as well as tumor cells.

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Supporting information

Materials and methods

Chemicals

The phospholipids 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-PEG_{2000} (DSPE-PEG_{2000}) were purchased from Lipoid (Ludwigshafen, Germany). Phosphatidylethanolamine-dioleoyl-sulforhodamine B (Rho-PE),1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3 benzoxadiazol-4-yl) (NBD-PE) and the cationic lipid 1,2-dipalmitoyl-3-trimethylammonium-propane (DPTAP Chloride salt) were provided by Avanti Polar Lipid Inc. Carboxyfluorescein was purified by recrystalization. [32]

Preparation of TSL

CTSL contained DPPC: DSPC: DPTAP: DSPE-PEG_{2000} in a molar ratio 60:25:10:5. NCTSL were composed of DPPC: DSPC: DSPE-PEG_{2000} in a molar ratio 70:25:5. Small TSL were prepared by lipid film hydration and extrusion method.[22] All lipids were dissolved in chlorophorm and methanol (9:1 vol/vol).Rho-PE was added at 0.1 mol % for fluorescent labelling of lipid bilayer. TSL used for flow cytometry contained 0.1% of NBD-PE in the lipid bilayer. The solvent was subsequently evaporated under vacuum in rotary evaporator until homogeneous lipid film was formed. The lipid film was hydrated in 100 mM CF solution, pH 7.2 at 60 °C for 30 min. The newly formed multilammelar vesicles were extruded subsequently 5 times through 200 nm, 100 nm, 80 nm and 50 nm polycarbonate filter (thermo barrel extruder at 60 °C) and resulted in small sized TSL. Nonentrapped CF was removed from liposomal CF by gel permeation chromatography using a PD-10 Sephadex column (GE Healthcare, Buckinghamshire, UK), eluted with HEPES buffer, pH 7.4 (10 mM HEPES, 135 mM NaCl). Size, polydispersity index (PDI) and zeta potential (ζ) were measured by dynamic light scattering using Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK).For the size and PDI measurements TSL were diluted in HEPES, pH 7.4, while the zeta potential was obtained in HEPES, pH 7. Lipid concentration was determined by phosphate assay.[33]

Differential scanning calorimetry

T_m of TSL was measured by differential scanning calorimetry (DSC) [34] using Capillary Cell MicroCalorimeter (MicroCal VP-DSC). Samples were scanned from 20-60 °C and then cooled to 20 °C with a heating and cooling rate of 1 °C/min. The data were

normalized to the maximum Cp (heat capacity) and the average of three independent batches of liposomes was plotted.

Stability in serum and encapsulation efficiency

Stability of TSL was tested by incubating samples of TSL suspension (1mM [lipid]) in pre-heated 90% fetal calf serum (FCS) (1:9 v/v) for 1h at 37 °C using a thermal-shaker (Eppendorf Thermomixer) at 300 rpm. Samples without incubation were considered as a blank (Io). Maximum CF fluorescence (positive control - I_∞) was achieved when incubating TSL suspension (1mM [lipid]) in 2% Triton X-100 in H_2O for 30 min in a thermoshaker at 55 °C and 1400 rpm. All samples were diluted in 10 mM Tris/NaCl 0.9%, pH 8.0 at 1:50 (v/v) (It) after incubation and CF fluorescence was measured by fluorimetry at Ex. 493 nm / Em. 513 nm (Hitachi F-4500 Fluorescence Spectrophotometer). Encapsulated CF was quantified as encapsulation ratio (CF/lipid)=[CF]/[lipid]. CF release was determined as CF (%) = (It−Io) / (I_∞−Io) × 100. Stability of TSL was calculated as 100 – CF (%).

Temperature-dependent CF release

Temperature-dependent release profile of CF from NCTSL and CTSL was established by fluorometry upon incubating the TSL samples (section 2.4) at various temperatures between 37- 45 °C for 5 min. The CF release (%) was calculated as in section 2.4.

Time-dependent CF release

Time-dependent release profile of CF from NCTSL and CTSL was performed at 42 °C. TSL suspension (1mM [lipid]) was mixed with pre-heated FCS (1:149 v/v) under stirring and CF release was measured over time (at 1, 2, 3, 4, 5, 30, 60 min). TSL samples without heating were considered as a blank. TSL were destroyed by adding 10% Triton X-100 (150:1 v/v) and considered as a positive control. The CF release (%) was calculated as in 2.4.

Cell culture

Tumor cell line BLM (human melanoma) was cultured in a Dulbecco's Modified Eagles' medium (Lonza, Belgium) containing 10% FCS. Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords and cultured in Human Endothelial SFM medium (Gibco, Invitrogen) enriched with 30% FCS. Cells were subcultured once a week using Trypsin (Sigma, Aldrich) and maintained at 37 °C, 5% CO_2 in a humidified incubator. All experiments were performed at a confluence of 80-90%.

Flow cytometry

Binding of TSL to BLM and HUVEC cells was assessed by flow cytometry (FACS) analysis. $1x10^5$ cells in suspension were incubated for 1h either at 4 °C or at 37 °C with various concentrations (100, 200, 500, 1000, 2000, 5000 nmol/ml) of NBD-PE labeled TSL. After incubation, cells were washed three times with medium with FCS to remove unbound liposomes. Liposomal NBD-PE fluorescence was determined at excitation and emission wavelengths of 470 and 530 nm by a BD FACScan (Becton Dickinson, San Jose, CA, USA). Data were analyzed with FlowJo software. From the mean fluorescent intensity data, the maximum, which was achieved at the highest liposome concentration, was set at 100% and further data were calculated as percentage of maximum mean fluorescent intensity (% of MFI). Ten thousand gated events were acquired per sample and samples were prepared in duplicate. Experiments were repeated at least three times with different batches of liposomes.

Living cell confocal microscopy

BLM or HUVEC cells were seeded at a concentration of 60000 cells/ml in cell culture chambers containing a cover glass insert coated with 0.1% gelatine or fibronectin, respectively. Cells were allowed to grow for 24 hours in the incubator. After 24h, BLM were incubated with 1000 nmol/ml CTSL for 1h at 4 °C or 37 °C and HUVEC for 15 min at 4 °C. After incubation, cells were washed 3 times with either DMEM (for BLM) or HUVEC medium (for HUVEC) with FCS. Cells were analyzed on a Zeiss LSM 510 META confocal laser scanning microscope. CF release was detected by 513 nm argon laser and Rho-PE was monitored by a 543 nm Helium –Neon laser. Images were taken from 37 °C to 43 °C for 1h (40 x objective lens 5 µm pinhole). Images of 1024 x 1024 pixels were analyzed using Zeiss LSM image software (Zeiss, Germany).

Animal models

B16BL6 (murine melanoma) and LLC (Lewis-lung carcinoma) cells were cultured in DMEM medium with 10% FCS. Ten million tumor cells were injected subcutaneously in the flanks of C57Bl6 mice and bulk tumors of 10 mm in diameter were used for transplantation into C57Bl6, expressing an eNos-tag-GFP fusion protein constitutively in their vascular endothelium. Tumor pieces were implanted in a dorsal skin flap window chamber for intravital imaging.[18, 35] Bulk mice were housed at 20-22 °C, humidity of 50-60%. Window chamber-bearing mice were used for experiments after 8-12 days of tumor implantation when tumor size reached 4-6 mm in diameter. These mice were housed in an incubator room with a humidity of 70% and temperature of 30-32 °C. NMRI nu/nu mice were used for observing possibility of CTSL binding to blood cells. They were housed at 20-22 °C and humidity of 50-60%. All mice were fed a standard laboratory diet *ad libitum* (Hope Farms Woerden, the Netherlands). Mice weighing 20-25 g were used for experiments. All animal experiments were performed in

compliance with protocols approved by the committee on Animal Research of the Erasmus MC, Rotterdam, The Netherlands.

In vivo binding to angiogenic endothelial cells and tumor cells

In vivo binding of CTSL and NCTSL to tumor and endothelial cells was observed by intravital confocal microscopy on dorsal skin-fold window chamber-bearing mice implanted with B16BL6 or LLC tumors. The mice were anesthetized with isoflurane (Nicholas Piramal, London, UK) and placed on a heated stage (37 °C) under the confocal microscope. Thermocouples (point-welded thin manganese and constantane wires, H. Drijfhout & Zoon's edelmetaalbedrijven, Amsterdam) were inserted in the window chamber for online monitoring of tissue temperature. A circular resistive heating coil, attached to the glass at the back of the chamber was used to provide homogeneous temperature distribution in the tissue. CTSL or NCTSL were injected i.v. through the penile or tail vein at a dose of 5 µmol. Binding of CTSL or NCTSL to vascular angiogenic endothelial cells or tumor cells was observed by confocal microscopy (Zeiss LSM 510 META) during 3h. Imaging started right after the injection and Rho-PE labeled liposomes were detected as described above (section 2.9). Mice were observed up to 24h after injection of TSL in order to visualize remaining bound liposomes to the endothelial and tumor cells. Images were analyzed as described in 2.9.

In vivo CF release upon hyperthermia

After 2h of binding and liposome extravasation, tumor was heated at 43 °C for 1h and CF release was detected as above (20x objective lens). Regions of interest were selected before and in the end of the hyperthermia treatment. Images were analyzed as described in 2.9.

Binding of CTSL to blood cells

Ten µmol of CTSL were injected through the tail veins of nude mice. After 2h, blood was withdrawn by heart puncture. Blood was spun down two times for 5 min at 400 g and blood cells were washed with medium containing FCS. After washing, the blood cells were placed on glass slides and covered with glass coverslips. Images were taken with Zeiss 100 M microscope, 63x objective lens.

Image inalysis

The mean fluorescent intensity (AU) from the green and the red channels at 37 °C and 43 °C was quantified for 14 positions obtained from 3 mice. The data are presented as average of the mean fluorescent intensity from the all positions with SEM. The data were analyzed by Image J software.

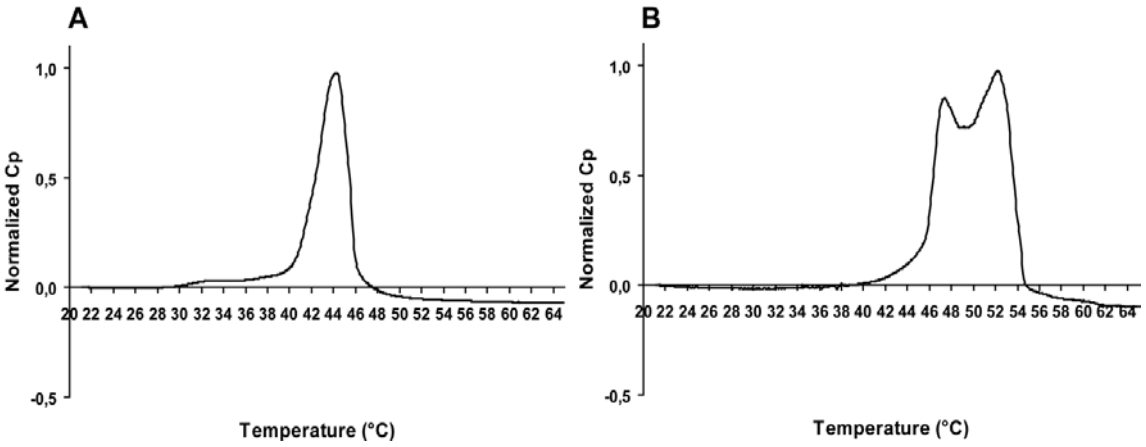

Fig 1. DSC analysis of NCTSL (A) and CTSL (B) in HEPES buffer, pH 7,4. The heat flow is 1 °C/min. Values represented in the figure show the normalized Cp as average from three independent batches of liposomes.

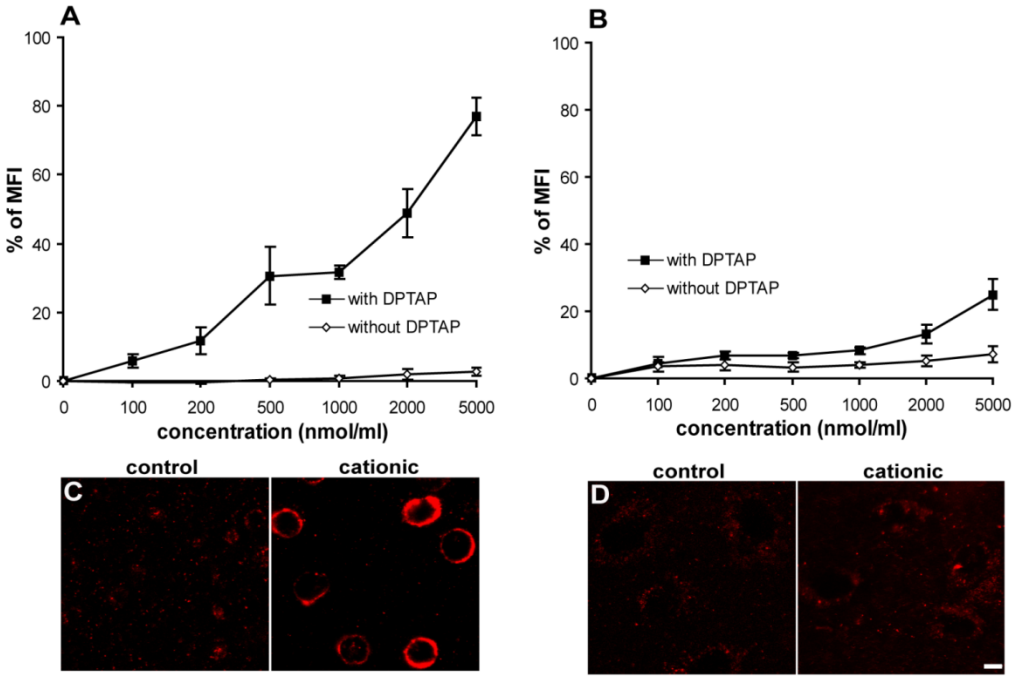

Fig 2. Binding of CTSL or NCTSL at 4 °C to BLM melanoma cells (A) and endothelial cells (HUVEC) (B) and confocal microscopy of BLM cells (C) and HUVEC (D). Cells in suspension were incubated for 1h with NBD-PE labeled CTSL or NCTSL at 4 °C. After washing, the NBD-PE fluorescent intensity was determined by flow cytometry. Values depicted in the figure represent the mean fluorescent intensity calculated as a percentage of the maximum mean fluorescent intensity from three experiments with

three different batches of liposomes. C and D. BLM or HUVEC cells were incubated with Rho-PE labeled liposomes (red color) for 1h at 4°C. After incubation, cells were washed and visualized by confocal microscopy. Scale bar applies for all images, 10 µm.

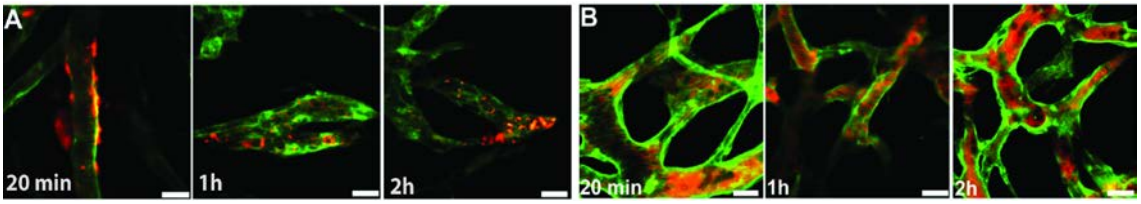

Fig 3. Intravital evaluation of binding of CTSL (A) or NCTSL (B) to angiogenic endothelial cells in LLC tumor model using the dorsal skin-fold chamber. Mice were injected with 5 µmol of lipid. Appearance of vasculature bound cationic liposomes started 20 min after injection and remained visible at least up to 2h. NCTSL hardly bound to the endothelial lining of tumor associated blood vessels. Scale bar applies for all images, 20 µm.

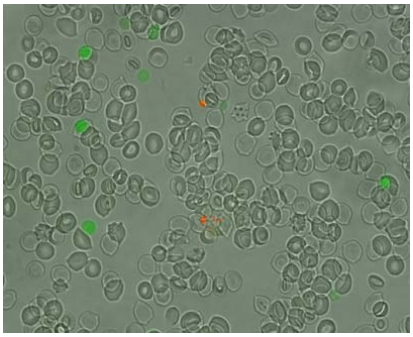

Fig 4. Binding of CTSL to blood cells in circulation. Ten µmol liposomes were injected in nude mice and blood was withdrawn 2h after injection. Images showing absence of CTSL binding to blood cells were taken by fluorescence microscopy.

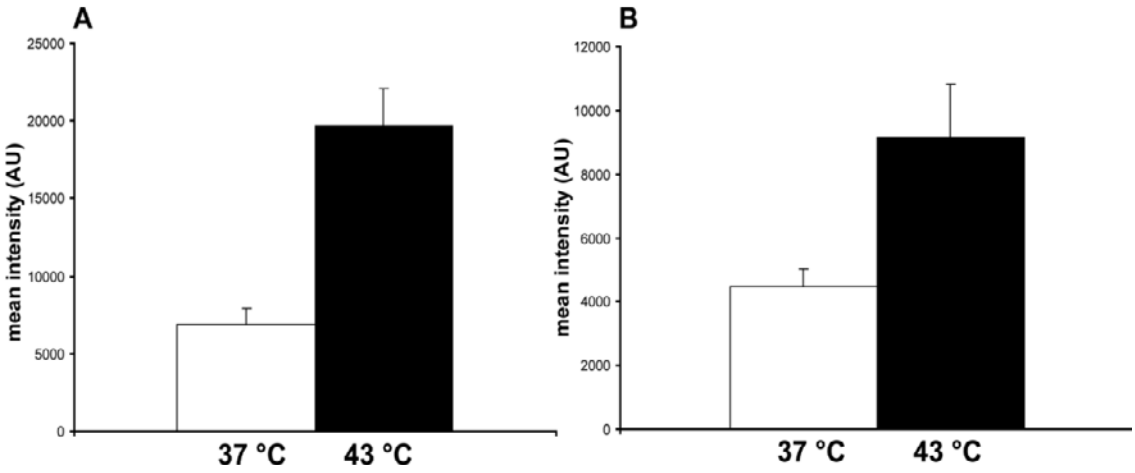

Fig 5. Effect of hyperthermia on CF release (A) and liposome binding to and extravasation from tumor vasculature (B). Columns, mean; bars, SEM.

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Chapter 3

Targeted and heat-triggered doxorubicin delivery to tumors by dual targeted cationic thermosensitive liposomes

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Abstract

Liposomal nanoparticles can circumvent toxicity of encapsulated chemotherapeutic drugs, but fall short in tumor-specific and efficient intracellular drug delivery. To overcome these shortcomings, we designed a multifunctional dual targeted, heat-responsive nanocarrier encapsulating doxorubicin (Dox) as a chemotherapeutic content. Dox-loaded cationic thermosensitive liposomes (Dox-CTSL) carry targeting functions addressing tumor cells and tumor vasculature and have a heat-responsive lipid bilayer. Targeted Dox-CTSL demonstrated superior uptake by and toxicity to different tumor cell lines and endothelial cells compared to non-targeted TSL. Heat-triggered intracellular Dox release in acidic cell compartments was visualized as fluorescent Dox nanobursts by live cell confocal microscopy. In vivo, using high resolution intravital microscopy, we demonstrated that Dox-CTSL upon an external heat-trigger delivered 3-fold higher Dox quantity to tumors than TSL. Dox-CTSL bound specifically to tumor vasculature, which in combination with the heat-triggered drug release caused significant tumor vessel damage, which was not observed when non-targeted TSL were administered. Therefore, Dox-CTSL have strong potency to increase drug efficacy due to targeted delivery and heat-triggered drug release in tumors.

Keywords: Cationic thermosensitive liposomes, hyperthermia, triggered drug release, cytotoxicity, cancer chemotherapy

1. Introduction

Liposomes currently represent one of the best studied nanoparticle-based drug delivery systems used for treatment of cancer and have several advantages over free drug administration. [1-3] Their small size (< 100nm) and prolonged presence in systemic circulation allow them to preferentially deliver chemotherapeutics to tumors by altering their biodistribution. [4] Due to these advantages, liposomal encapsulation has significantly decreased toxicity of various chemotherapeutic drugs. [5-7] Yet, mainly limited improvements in therapeutic efficacy were demonstrated. [6, 8] The latter is due to a low retention of liposomes in tumors and their high intrinsic stability causing low concentrations of drug becoming bioavailable in tumor tissue at a slow pace. [9-11] The aim of this study is to develop a drug delivery strategy that deals with these short-comings in current clinical liposomal chemotherapy. Our strategy employs a combination of tumor targeting of drug loaded nanoparticles with an externally triggerable controlled release function. To achieve this we encapsulate drugs into targeted cationic liposomes with a thermosensitive bilayer. This novel nanoparticle makes use of shielded cationic charged lipids to induce specificity for tumor vasculature and tumor cells thereby increasing nanoparticle tumor retention, in combination with a thermosensitive liposomal bilayer for heat-triggered drug release.

Tumor angiogenesis is an important mechanism for supply of nutrients and oxygen to tumor cells. Therefore, attacking tumor vasculature is a promising way of treating cancer. For nanoparticle-based drug delivery, tumor pathophysiological barriers such as the vascular endothelial barrier and high interstitial fluid pressure [12] appear as

obstacles for effective drug delivery. [13] Vasculature targeting of nanoparticles may overcome these barriers through preferential delivery of chemotherapeutics to angiogenic endothelial cells and may provide a promising alternative strategy. [14, 15] Next to vascular targeting, such nanoparticles may benefit from the somewhat more leaky tumor vasculature allowing for their extravasation into tumor interstitium and targeting of tumor cells. [16]

Cationic liposomes have been described to selectively target angiogenic endothelial cells and tumor cells based on electrostatic interactions between their cationic surface and overexpressed anionic molecules on angiogenic endothelial and tumor cell membranes. [17,14, 18, 19] In addition, coating cationic liposomes with PEG will prolong their circulation half-life, while preserving their targeting capacity. [12] Slow and irregular blood flow in tumors further contributes to strong binding of cationic liposomes to tumor vasculature [17], whereas extravasation allows tumor cell targeting. [20] Upon binding to either tumor endothelial or tumor cells, the cationic charge can promote nanoparticle internalization, bringing the drug closer to its active site intracellularly. The use of thermosensitive bilayers and hyperthermia is known to trigger rapid drug release from nanoparticles locally in heated tumor areas. [20-24] Combining the dual targeting functionality and internalization properties of cationic liposomes with a heat-triggerable release function can ensure controlled release of drug contents at the tumor site either extra- or intracellularly. Next to triggering release, HT also plays a key role in modifying tumor microenvironment to increase liposomal drug delivery to tumors. [22] Temperature of 41-43 °C is known to increase tumor blood flow, oxygenation and vascular permeability [22, 25] and specifically increases permeability to liposomes. [25-27]

Solid efforts have been made to design liposomes which are either thermosensitive or targeted. [28-31] Thermosensitive liposomes can be obtained by incorporating lipids in the bilayer that undergo a gel-to-liquid phase transition at elevated temperatures causing release of the encapsulated drug. [32, 33] We have recently developed a cationic thermosensitive liposome (CTSL) formulation, which promoted binding and internalization into tumor and angiogenic endothelial cells in comparison to thermosensitive liposomes (TSL) and proved to be thermosensitive in vitro and in vivo by releasing a liposome- encapsulated fluorescent marker upon heat. [20] Based on this novel dual targeted thermosensitive nanoparticle we designed a variant containing the widely used anti-cancer drug doxorubicin.

In this study we report on the development, characterization and application of doxorubicin loaded targeted thermosensitive liposomes. CTSL were applied in combination with mild hyperthermia to different tumor cells and endothelial cells in order to test their cytotoxic potential. In addition, we studied the intracellular fate of the nanocarrier and its drug content by live cell imaging. Finally, we investigated the effect of mild hyperthermia on intratumoral fate, endothelial targeting and extravasation of CTSL, its drug delivery potential and its subsequent damaging effects on tumor vasculature using high resolution intravital microscopy on dorsal skin-fold window chamber bearing mice implanted with tumors.

2. Materials and methods.

2.1. Chemicals. The phospholipids 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-PEG2000 (DSPE-PEG2000) were purchased from Lipoid (Ludwigshafen, Germany). 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3 benzoxadiazol-4-yl) (NBD-PE), phosphatidylethanolamine-dioleoyl-sulforhodamine B (Rho-PE) and the cationic lipid 1,2-dipalmitoyl-3-trimethylammonium-propane (DPTAP Chloride salt) were from Avanti Polar Lipid Inc. Doxorubicin-HCl was purchased from Pharmachemie (Haarlem, The Netherlands). Sodium 3'-[(1-phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) was provided by Sigma- Aldrich (Zwijndrecht, The Netherlands). LysoTracker Red DND-99 and Dioctadecyl tetramethylindotricarbocyanine perchlorate (DiD-C_{18}(3)) were provided by Invitrogen.

2.2. Preparation of TSL. CTSL were composed of DPPC: DSPC: DPTAP: DSPE-PEG2000 in a molar ratio 60:25:10:5 (CTSL-10) or 62.5:25:7.5:5 (CTSL-7.5). NCTSL consisted of DPPC: DSPC: DSPE-PEG2000 in a molar ratio 70:25:5. All the liposomes were prepared by lipid film hydration and extrusion method.[34] The lipids were dissolved in chloroform and methanol (9:1 vol/vol). TSL used for confocal microscopy contained 0.3 mol% of NBD-PE in the lipid bilayer and TSL used for intravital microscopy contained 0.1 mol% of Rho-PE or 0.3 mol% of DiD. The solvent was subsequently evaporated under vacuum in rotary evaporator until homogeneous lipid film was formed. The lipid film was hydrated in 250 mM $(NH_4)_2SO_4$ solution at 60 °C for 30 min. The newly formed multilammelar vesicles were extruded subsequently 5 times through 100 nm and 10 times through 50 nm polycarbonate filter (thermo barrel extruder at 60 °C) and resulted in small sized TSL. Extraliposomal $(NH_4)_2SO_4$ was removed from liposomal suspension by gel permeation chromatography using a PD-10 Sephadex column (GE Healthcare, Buckinghamshire, UK), eluted with HEPES buffer, pH 7.4 (10 mM HEPES, 135 mM NaCl). Size, polydispersity index (PDI) and zeta potential (ζ) were measured by dynamic light scattering using Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). For size and PDI measurements, TSL were diluted in HEPES, pH 7.4, while the zeta potential was obtained in HEPES, pH 7 without NaCl. Lipid concentration was determined by phosphate assay. [35] After the phosphate concentration was determined, doxorubicin was loaded into the liposomes (5mM lipid) in 0.05:1 drug:lipid ratio (mol:mol) at 38 °C for 1h. The liposomes were concentrated by ultracentrifugation for 2h, 4°C, 49000 rpm for control and 29000 rpm for cationic liposomes. The pellet was resuspended in HEPES buffer, pH 7.4 and left overnight on slow rotation at 4 °C. Then the liposomes were passed through PD 10 column eluted with HEPES buffer, pH 7.4 to remove residual nonentrapped doxorubicin. Doxorubicin concentration was measured by spectrophotometer at Ex. 479 nm / Em. 590 nm.

2.3. TEM cryo imaging. Samples for imaging were prepared by applying a 2 µl droplet of liposome suspension to a lacy carbon film and subsequently plunge-freezing this sample into liquid ethane using a Vitrobot. An amorphous ('vitrified') ice film which contains the particles of interest was created. Cryo-TEM studies were performed using a FEI TECNAI F30ST (300kV, using a cryo-holder, keeping the sample at –174 ºC during the studies). Imaging was done in low-dose mode on a CCD camera (1 k x 1k). Due to the negatively charged grid used for sample holding, cationic liposomes were attracted by the grid to some extent. Therefore, a lower number of cationic liposomes as compared to the noncationic ones was visualized in positions between the grids. Observations on both locations indicated no morphological differences between the liposomes.

2.4. Stability at physiological conditions and prolonged storage at 4 °C. Stability of TSL was established by incubating 10mM [lipid]) in pre-heated FCS (1:149 v/v) under stirring and Dox release was measured for 1h at 37 °C. Samples without incubation were considered as a blank (Io). TSL were destroyed by adding 10% Triton X-100 (150:1 v/v) and considered as a positive control (I_{∞}). Fluorescence was measured by fluorometry at Ex. 479 nm / Em. 590 nm (Hitachi F-4500 Fluorescence Spectrophotometer). Dox release was determined as Dox (%) = (It−Io) / (I_{∞}−Io) × 100. Stability of TSL was calculated as 100 – Dox (%). Liposomes were also tested for prolonged storage over 4 weeks at 4 °C by measuring size, pdi and dox release. Dox release measurements were performed by mixing 1mM [lipid]) with 10 mM Tris/NaCl 0.9%, pH 8.0 at 1:149 (v/v) and dox fluorescence was measured at Ex. 479 nm / Em. 590 nm. TSL were destroyed by adding 10% Triton X-100 (150:1 v/v) and considered as a positive control and dox release measured as mentioned above.

2.5. Temperature-dependent dox release. Temperature-dependent dox release profile from NCTSL and CTSL was performed by fluorometry upon incubating the TSL samples (10mM [lipid]) in pre-heated 90% fetal calf serum (FCS) at various temperatures between 37- 45 °C for 5 min in a thermal-shaker (Eppendorf Thermomixer) at 300 rpm. Maximum dox fluorescence (positive control) was achieved when incubating TSL suspension (10mM [lipid]) in 2% Triton X-100 in H_2O for 30 min in a thermal shaker at 55 °C and 1400 rpm. All samples were diluted in 10 mM Tris/NaCl 0.9%, pH 8.0 at 1:50 (v/v) after incubation and dox fluorescence was measured by fluorometry at Ex. 479 nm / Em. 590 nm. The dox release (%) was calculated as in section 4.

2.6. Time-dependent dox release. Time-dependent dox release from NCTSL and CTSL was established at 42 °C. TSL suspension (10mM [lipid]) was mixed with pre-heated FCS (1:149 v/v) under stirring and dox release was measured over time (at 1, 2, 3, 4, 5, 30, 60 min). TSL samples without heating (RT) were considered as a blank. TSL were destroyed by adding 10% Triton X-100 (150:1 v/v) and considered as a positive control. The dox release (%) was calculated as in 4.

2.7. Cell culture. Tumor cell lines BLM (human melanoma), B16 (murine melanoma) and LLC (Lewis lung carcinoma) were cultured in a Dulbecco's Modified Eagles' medium (Invitrogen) containing 10% FCS. Tumor cell line BFS 1 (murine sarcoma) was cultured in RPMI medium containing 10% FCS. Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords and cultured in Human Endothelial SFM medium (Gibco, Invitrogen) enriched with 30% FCS. Cells were subcultured once a week using Trypsin (Sigma, Aldrich) and maintained at 37 °C, 5% CO_2 in a humidified incubator. All experiments were performed at a confluence of 80-90%, except for cytotoxicity experiments where the confluence was reched after 72h.

2.8. Cell toxicity. BLM, B16, BFS 1, LLC and HUVEC cells were plated in 96 well plates at concentration 10000cells/well for BLM, BFS 1 and LLC, 12000cells/well for B16 and 6000/well for HUVEC. The cells were allowed to adhere to the bottom of the wells for 24h and after that incubated with various concentrations of free Dox or Dox incorporated in either TSL or CTSL for 1h at 37 °C. After 1h, liposomes were removed and cells washed 3 times with DMEM (for BLM, B16 and LLC) or RPMI (for BFS 1) or HUVEC medium (for HUVEC) without FCS. Plates were placed at either 37 °C or 42 °C for 1h and then left in the incubator at 37 °C for 72h. Cell survival was determined by XTT assay. Electron coupling reagent *N*-methyl dibenzopyrazine methylsulfate (1.25 mM in PBS; Sigma) (100 µl) was mixed with 5ml of XTT solution (1 mg/ml in RPMI 1640) and cells were incubated with the mixture for 1h at 37 °C. Afterwards, XTT conversion was measured at 490 nm in a PerkinElmer Victor Wallac plate reader (Perkin Elmer, Groningen, The Netherlands). IC50 (the concentration of drug causing 50% reduction of the survival of the control) was calculated from the survival growth curves by fitting using GraphPad Prism software (GraphPad, San Diego, CA, USA).

2.9. Fluorescence microscopy. BLM, BFS 1, LLC, B16 or HUVEC cells were seeded at a concentration of 100000 cells/ml in cell culture chambers containing a cover glass insert coated with 0.1% gelatine. Cells were allowed to grow for 24 hours in the incubator. After 24h, cells were incubated with Dox-TSL or Dox-CTSL at 50 µM Dox for 1h at 37 °C. After 1h of incubation, liposomes were removed, cells washed 3 times with medium without FCS and then substituted with fresh medium with FCS. Images were taken with a fluorescence microscope (Zeiss Axiovert 100M equipped with Hamamatsu C 4742-98 camera) after 1h at 37 °C or 42 °C (63x/NA1.40 objective lens).

2.10. Live cell confocal microscopy. BLM, BFS 1, LLC, B16BL6 or HUVEC cells were seeded at a concentration of 100000 cells/ml in cell culture chambers containing a cover glass insert coated with 0.1% gelatine. Cells were allowed to grow for 24 hours in the incubator. After 24h, cells were incubated with 1000 nmol/ml NBD-PE labelled

CTSL for 1h at 37 °C and for 30min with lysotracker (LysoTracker® Red DND-99, Molecular Probes). After incubation, cells were washed 3 times with medium without FCS. Cells were analysed on a Zeiss LSM 510 META confocal laser scanning microscope. NBD-PE fluorescence was detected by 488 nm argon laser and lysotracker was monitored by a 543 nm Helium –Neon laser. Images were taken at 37 °C for 1h. For Dox release experiments, cells were also incubated with NBD-PE labelled Dox-CTSL-7.5 for 1h at 37 °C and after that washed 3 times with medium without FCS. Dox release was followed in time for 1h at 42 °C and its fluorescence was detected by a 543 nm Helium –Neon laser, pinhole 2,5 µm. Images of 1024 x 1024 pixels were analysed using Zeiss LSM image software (Zeiss, Germany) (40x/NA1.30 objective lens 2,5 µm pinhole).

2.11. Doxorubicin extraction from tumor and endothelial cells. BLM, BFS 1, LLC, B16 or HUVEC cells were seeded at a concentration of 4.10x5 cells/well in 6 well plates. The cells were allowed to adhere to the bottom of the wells for 24h and afterwards incubated with 100 µM Dox-CTSL or Dox-TSL for 1h at 37 °C. After incubation, cells were washed three times with medium without FCS to remove unbound liposomes and then incubated at either 37 °C or 42 °C for 1h. Cells were then trypsinized and cell suspensions containing the same amount of cells prepared. Afterwards, cells were centrifuged at 1400g for 10 min. Supernatant was discarded and the pellet was resuspended in premixed Acidic isopropanol with 4% Triton X-100 (1:1 v/v). The samples were sonicated in a bath sonicator for 45 min. The concentration of dox was measured by fluorometer at Ex. 479 nm / Em. 590 nm.

2.12. Animal models. B16 (murine melanoma) cells were cultured in DMEM medium with 10% FCS. Ten million tumor cells were injected subcutaneously in the flanks of C57Bl6 mice and bulk tumors of 10 mm in diameter were used for transplantation into C57Bl6, expressing an eNos-tag-GFP fusion protein constitutively in their vascular endothelium. Tumor pieces were implanted in a dorsal skin flap window chamber for intravital imaging. Bulk mice were housed at 20-22 °C, humidity of 50-60%. Window chamber-bearing mice were used for experiments after 8-12 days of tumor implantation when tumor size reached 4-6 mm in diameter. These mice were housed in an incubator room with a humidity of 70% and temperature of 30-32 °C. All mice were fed a standard laboratory diet *ad libitum* (Hope Farms Woerden, the Netherlands). Mice weighing 20-25 g were used for experiments. All animal experiments were performed in compliance with protocols approved by the committee on Animal Research of the Erasmus MC, Rotterdam, The Netherlands.

2.13. In vivo extravasation of CTSL. In order to determine the effect of hyperthermia on liposome extravasation, intravital microscopy on dorsal skin-fold window chamber-bearing mice implanted with B16 tumor was performed. The mice were anesthetized with isoflurane (Nicholas Piramal, London, UK) and placed on a heated stage (37 °C)

under the confocal microscope. Thermocouples (point-welded thin manganese and constantane wires, H. Drijfhout & Zoon's edelmetaalbedrijven, Amsterdam) were inserted in the window chamber for online monitoring of tissue temperature. A circular resistive heating coil, attached to the glass at the back of the chamber was used to provide homogeneous temperature distribution in the tissue. Rho-PE or DiD- labelled CTSL liposomes were injected through the penile or tail vein and left to circulate in the blood stream for 2 or 5h respectively. After 2 or 5h, the tumor tissue of B16 window chamber-bearing mice was heated at 42 °C for 1h. Images were recorded during the 1h of heat triggering in order to detect its effect on extravasation, which may promote higher delivery of liposomes to tumor cells. Representative images of 1024 x 1024 pixels were analysed using Zeiss LSM image software (Zeiss, Germany) (10x/NA0.30 objective lens).

2.14. In vivo dox release upon hyperthermia and uptake by tumor and angiogenic endothelial cells. In order to evaluate Dox release during hyperthermia and its uptake by tumor vascular endothelial cells and tumor cells, Dox-CTSL or Dox-TSL were injected i.v. through the penile vein at a dose of 5mg/kg doxorubicin. CTSL or TSL were allowed to circulate for 5h at body temperature in order to be able to bind to vascular angiogenic endothelial cells or tumor cells and observed by confocal microscopy (Zeiss LSM 510 META). After 5h of circulation, tumor was heated at 42 °C for 1h and release and uptake was detected as above (10x/NA0.30 objective lens). Regions of interest were selected before, during and at the end of the hyperthermia treatment. Mice were observed up to 72h after injection of liposomes in order to visualize Dox and liposome retention in the tumor and observe possible vessel destruction. Images of 1024 x 1024 pixels were analysed using Zeiss LSM image software (Zeiss, Germany).

2.15. In vivo Dox and liposome quantification. The integrated density (IntDen) from either the red (Dox) or the purple (DiD) channel (obtained after setting a threshold) representing released doxorubicin at 42 °C and liposomes respectively, was quantified by Image J software. Mice were injected with either CTSL or TSL and Dox was quantified from 11 positions from each group, which were obtained from 3 mice. When DiD quantified, 5 or 6 positions were used per mouse obtained from either 5 mice (cationic) or 3 mice (control). The data are presented as an average of IntDen of all the positions of each mouse.

3. Results.

3.1. Liposome characterization

CTSL and TSL were composed of the same phospholipids and PEG-lipid; DPPC:DSPC and DSPE-PEG$_{2000}$. CTSL contained an additional amount of the cationic lipid DPTAP in replacement of a similar quantity of DPPC. All formulations were prepared

by lipid film hydration and extrusion method. [34] Dox was loaded after extrusion by $(NH_4)_2SO_4$ loading method. [36] Initially, CTSL were prepared with 10 mol % of DPTAP.[20] Empty CTSL-10 showed size below 100 nm and monodispersity (supporting information, table 1) similar to previous carboxyfluorescein-loaded CTSL. [20] However, after Dox loading these liposomes displayed inhomogeneous size distribution and appearance of a small second peak in the dynamic laser light scattering analysis, which indicates some aggregate formation (supplemental information, Fig 1A). As we thought the observed instability to be related to the cationic lipid, we lowered its amount to 7.5%. This resulted in disappearance of the second peak in DLS measurements and a homogeneous liposome formulation after Dox-loading (supplemental information, Fig 1B). After decreasing the amount of cationic lipid, we analysed particle characteristics and redefined the cellular binding capacity of the novel formulation in comparison to Dox-CTSL-10.

Liposomes were characterized by measuring size, polydispersity index (pdi), zeta potential (ζ) and Dox encapsulation efficiency. Moreover, the three formulations were tested for stability at storage conditions (4 °C) during 4 weeks. In order to mimic and evaluate liposome stability in circulation, Dox retention was analysed at 37 °C in full serum for 1h (Table 1). Dox-CTSL-7.5 and TSL represented smaller hydrodynamic diameter than Dox-CTSL-10, and also displayed a pdi <0,1, representing homogeneity. All formulations demonstrated optimal remote loading of doxorubicin (>90% encapsulation efficiency). When incubated in serum at 37 °C for 1 h, all liposome formulations retained >70% of the encapsulated Dox, with the highest level of Dox release from CTSL-10. Storage of liposomes at 4°C for 4 weeks proved both Dox-TSL and Dox-CTSL-7.5 were stable with regard to particle size, pdI and Dox retention. By contrast, Dox-CTSL-10 were less stable showing increased size and pdi after 4 weeks storage at 4 °C. All three liposome formulations released a minimal amount (10% or less) of Dox in 4 weeks at 4 °C (Table 1).

**Table 1. ***Characterisation of TSL, CTSL-7.5 and CTSL-10. Mean of three independent experiments. pdI:polydispersity index.

Batch	Size (nm)	pdI	Zeta potential (mV)	Encapsulation efficiency (%)	Entrapped Dox (%) in 99 % FCS for 1h at 37 °C	Size in 4 weeks at 4 °C (nm)	pdI in 4 weeks at 4 °C	Dox release (%) in 4 weeks at 4 °C
Dox-TSL	83±0.8	0.04±0.01	-10.5±0.5	>95	92±1.8	83.4±1.1	0.04±0.01	10.4±1.1
Dox-CTSL-10	99±3.2	0.13±0.03	11.4±0.7	>90	73±3.5	107.6±5.8	0.19±0.01	7.5±0.9
Dox-CTSL-7.5	84±2.0	0.05±0.01	6.0±0.4	>95	84±1.1	86.9±3.3	0.07±0.01	6.3±0.5

3.2. Temperature- and Time-dependent Dox release kinetics

Active loading of Dox by ammonium sulphate gradient method resulted in mostly spherical liposomal structures with intraliposomal Dox crystals, which were well visualized in TSL and CTSL-7.5 (Fig 1A, B). CTSL-10 displayed more aberrant structures next to spherical liposomes including elongated rod-shaped Dox-precipitate

containing particles, bilayer sheets and disc shaped micelles (DSM), confirming inhomogeneity observed by DLS (Fig 1C). A few DSMs were also observed in the images of CTSL-7.5, but not in TSL preparations (Fig 1A and B). These structures were not related to Dox loading as they were also observed before loading (Fig 2 supplemental).

In order to test thermosensitivity of all liposome formulations, temperature- and time-dependent release kinetics of Dox in presence of serum were measured. All three liposome formulations showed a clear temperature-dependent Dox release, which increased with temperature during a 5 min heating period (Fig 1D). All formulations were stable at 37-38 °C showing no Dox release in 5 min. All three liposome formulations showed initiation of Dox release at temperature of 39 °C, but their further temperature release profiles deviated. TSL and CTSL-7.5 displayed similar release kinetics up to 42 °C, after which Dox release from TSL declined similar to earlier observations [24] and Dox release from CTSL-7.5 remained high up to 45 °C with 90% release in 5 min. Dox release from CTSL-10 increased with increasing temperature until 45 °C, but was lower at all temperatures than the other formulations reaching maximally 60% release in 5 min at 45 °C.

Release kinetics in time at a constant temperature of 42 °C in 99% serum showed that all liposome formulations released increasing Dox levels in time (Fig 1E). The release kinetics for TSL and CTSL-7.5 were nearly similar, demonstrating fast release of 80% and 65%, respectively in the first minute. Dox release from both formulations continued to increase in time approaching maximal release after approximately 10 min. By contrast, CTSL-10 displayed slower release rate with 20% Dox release after 1 min reaching ~ 87% in 1h.

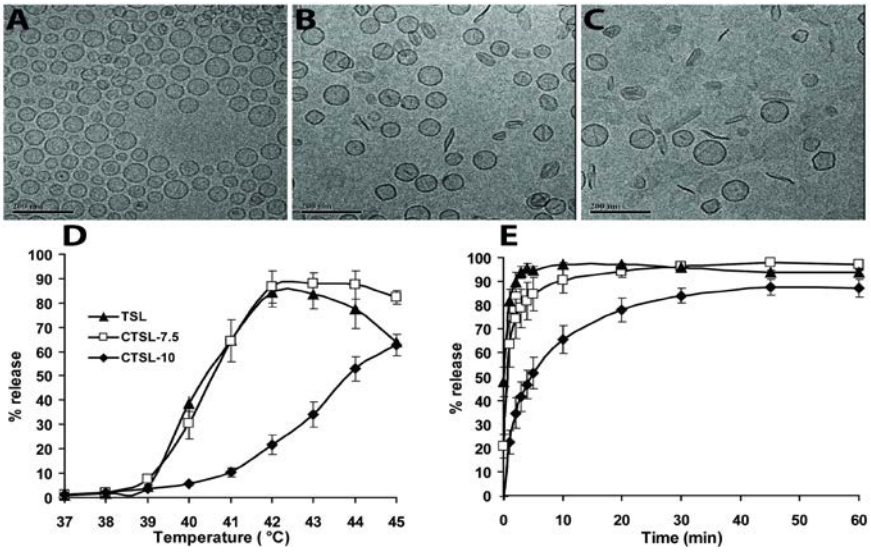

Fig 1. TEM cryo imaging of Dox-TSL (A), Dox-CTSL-7.5 (B) and Dox-CTSL-10 (C). Bar, 200nm. Temperature- (D) and time-dependent (E) Dox release from CTSL and

TSL. (D) Temperature-dependent release of Dox was measured upon a 5 min. exposure of TSL in 90% of serum to temperatures varying between 37−45 °C. (E) Time-dependent Dox release from TSL in 99% serum was measured at constant temperature of 42 °C at various time points up to 1 h. Mean ± SEM of three independent experiments with three independent batches of liposomes.

3.3. Dox uptake and release in tumor and endothelial cells

Triggered Dox release from CTSL at the cellular level was studied by live cell fluorescent microscopy on 4 different tumor cell lines (BLM, B16, BFS 1 and LLC) and human endothelial cells (HUVEC) (Fig 2A). Cells were incubated for 1h at 37 °C to allow binding and internalization of liposomes. After removing unbound liposomes, continued control incubation at 37 °C for 1h caused only minimal Dox release from the three different formulations in all cell lines proving significant liposome stability at physiological conditions. However, upon heating cells to 42 °C for 1 h increased intracellular Dox fluorescence was observed from cells incubated with CTSL compared to cells treated with TSL, representing cellular targeting of the CTSL and triggered intracellular Dox release. No differences were observed between CTSL-7.5 and CTSL-10. Dox delivery to HUVEC was less abundant compared to tumor cells, which is in accordance with earlier observations that cationic liposomes bind to a lesser extent to HUVEC cells than to tumor cells in vitro. [20]

Dox targeting was confirmed by measuring cellular Dox levels in tumor cells (BLM, B16, BFS 1, LLC) and HUVEC after a 1h incubation with liposomal Dox formulations followed by 1h HT at 42 °C or normothermia (NT) at 37 °C (Fig 2B). Cellular Dox delivery by CTSL was higher than TSL, confirming microscopy data. No significant differences between both CTSL were observed. Application of HT for 1h at 42 °C, which triggered Dox release from the nanoparticles, did not affect total cellular Dox levels in any of the cell lines showing that heat-released Dox remains intracellular.

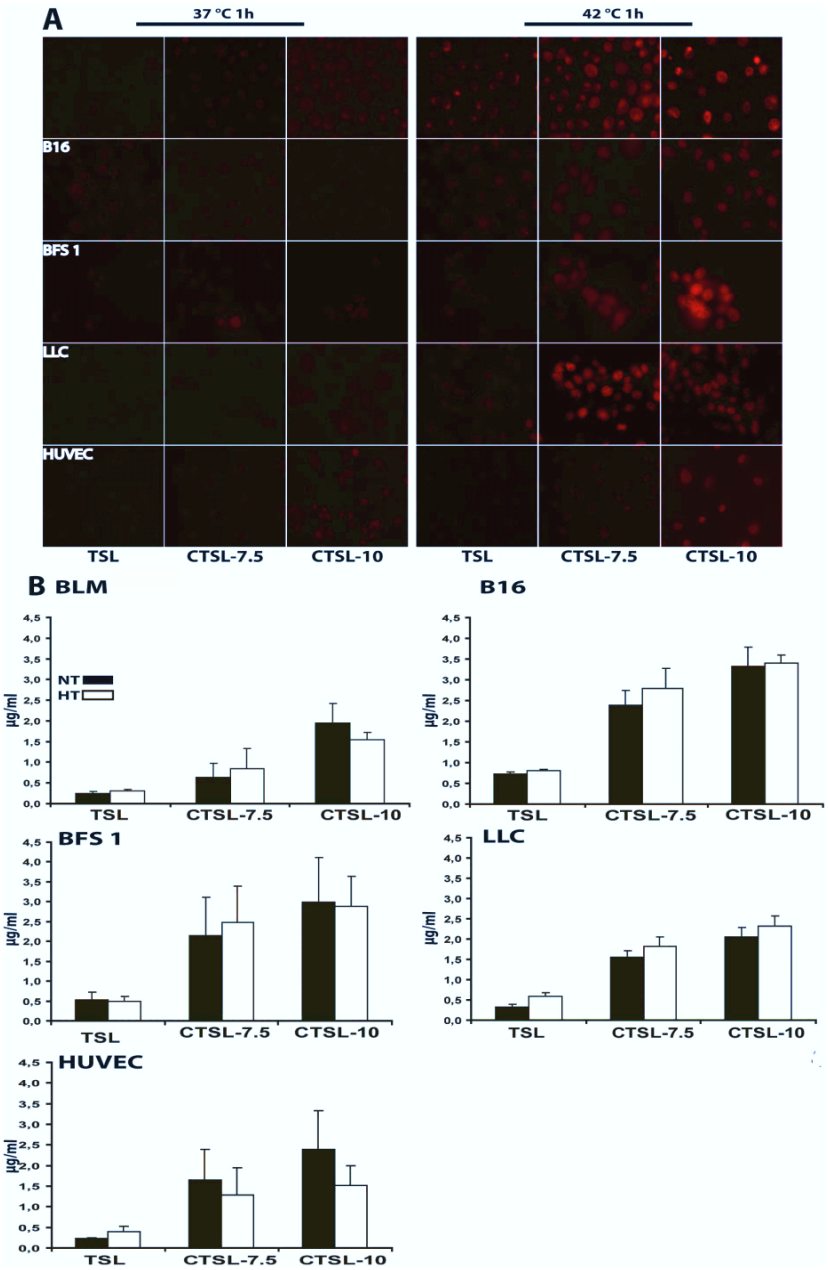

Fig 2. A, Targeted and triggered Dox delivery studied by live cell fluorescent microscopy on melanoma (murine B16 and human BLM), sarcoma (murine BFS1), Lewis lung carcinoma (murine LLC) and HUVECs. Cells were incubated for 1h at 37 °C with Dox-TSL, Dox-CTSL-7.5 or Dox-CTSL-10 at 50 µM Dox in order to allow binding and internalization of liposomes. After 1h, cells were washed 3x with wash medium to remove unbound liposomes. HT at 42 °C for 1h was applied to trigger doxorubicin release and compared to NT at 37 °C. B, Quantification of cellular

doxorubicin levels in BLM, B16, BFS 1, LLC tumor cells and HUVECs by Dox extraction method at 37 °C (black bars) and 42 °C (white bars). Cells were incubated with 100 µM liposomal Dox, after which they were washed to remove unbound liposomes and treated either at 37 °C or 42 °C for 1h. Cellular doxorubicin was extracted from cell lysates and measured fluorometrically. Mean ± SEM of three independent experiments with three independent batches of liposomes.

3.4. Cytotoxicity of Dox encapsulated in TSL, CTSL-7.5 or CTSL-10 upon NT and HT

A short (1h) treatment of BLM, B16, BFS 1, LLC tumor cells and HUVEC with Dox-CTSL and Dox-TSL demonstrated superior toxicity of Dox-CTSL to all cell lines as determined 72h later (table 2). Inhibitory concentrations of Dox killing 50% of the cells (IC50) were similar for both Dox-CTSL and 2-10 fold lower than Dox-TSL in the various cell lines, confirming that decreasing the amount of cationic lipid in the liposomal bilayer from 10 to 7.5 mol% did not interfere with cellular CTSL mediated Dox targeting and subsequent induction of cytotoxicity. Application of a 1h HT at 42 °C following CTSL incubation and removal could not further impact cytotoxicity outcome 72 h later.

As cellular Dox delivery and cytotoxicity displayed no differences between both Dox-CTSL formulations, CTSL-7.5 (from now on abbreviated as CTSL), which proved a pharmaceutically more stable formulation with faster drug release kinetics, was chosen for further experimentation.

Table 2. *IC 50 values in µM Dox of BLM human melanoma, B16 murine melanoma, BFS 1 murine sarcoma, LLC murine lung carcinoma and endothelial cell (HUVEC) treated with Dox formulated in TSL CTSL-7.5 or CTSL-10 for 1h at 37 °C. After removal of unbound liposomes, cells were subjected to HT at 42 °C for another 1h (grey rows) or NT (white rows) as control.

	IC 50 (µM)				
Treatment	**BLM**	**B16**	**LLC**	**BFS 1**	**HUVEC**
TSL	16.6 ± 8.1	99.1 ± 0.8	17.4 ± 7.4	11.4± 4.1	24.7 ± 6.4
Dox-CTSL-7.5	3.5 ± 0.7	11.7 ± 1.0	4.0 ± 1.1	2.9± 0.9	9.1 ± 4.1
Dox-CTSL-10	4.4 ± 1.5	9.8 ± 4.2	4.3 ± 1.6	3.3 ± 1.0	15.9 ± 8.1
TSL	16.3 ± 4.6	83.8 ± 8.3	14.3 ± 4.2	12.2± 5.8	32.2 ± 8.6
Dox-CTSL-7.5	4.1 ± 0.6	8.1 ± 1.6	3.2 ± 0.7	7.4± 3.1	14.5 ± 8.3
Dox-CTSL-10	3.5 ± 0.4	7.5 ± 2.5	3.2 ± 1.5	6.5 ± 3.5	22.5 ± 7.4

3.5. Uptake of CTSL in lysosomes

Next we investigated internalization and intracellular fate of CTSL labelled with a fluorescent NBD-PE phospholipid bilayer marker in all cell lines by live cell confocal microscopy. After 1h incubation, NBD-CTSL (green fluorescence) were observed at the cell surface and internalized in all 5 studied cell lines and appeared to colocalize to considerable extent with lysosomes stained with lysotracker (red fluorescence) (Fig 3). Besides lysosomal colocalization (yellow in merged images) also punctuate liposomal fluorescence (green) was observed in the cytoplasm in non-acidic cell organelles (magnified images in supporting information, Fig 3).

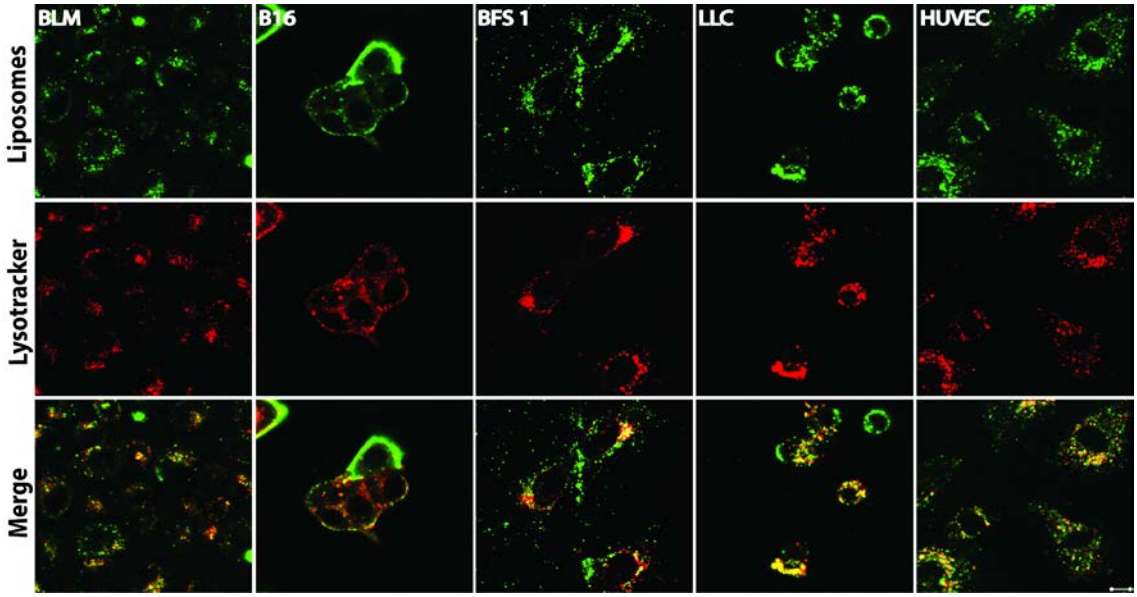

Fig 3. Confocal microscopy on tumor cells (BLM, B16, BFS1, LLC) and endothelial cells (HUVEC) incubated with NBD-PE labelled CTSL (green) for 1h at 37 °C and lysotracker (red). After 1h, unbound liposomes were removed by 3x washing with medium without FCS and live cell imaging was performed immediately at 37 °C. Liposomes were found cell-bound and internalized into the cytoplasm (green) and colocalized to some extent with the lysotracker (red) as visualized in yellow in the merged images. Images were taken by confocal microscope (40x, 2,5µm pinhole, 2x zoom). Scale bar applies for all images, 10 µm.

3.6. Intracellular, HT-triggered Dox release from CTSL in tumor and endothelial cells

To prove that CTSL deliver their drug payload intracellularly, live cell confocal microscopy was performed on tumor and endothelial cells incubated for 1h at 37 °C with Dox-NBD-CTSL (Fig 4). Dox is quenched inside CTSL and its red fluorescence appears only upon release. After removal of unbound liposomes, at 37 °C CTSL (green) were internalized and displayed minimal Dox release (red) in BLM, BFS1 and LLC. In

B16 and HUVEC, a higher intracellular and mostly nuclear doxorubicin fluorescence was observed upon Dox-CTSL incubation, likely representing faster cellular processing of the nanoparticles. A subsequent heat trigger at 42 °C immediately induced Dox release in all cell lines as is evident from appearance of cytoplasmic colocalization of released Dox from CTSL (yellow) and increases in nuclear Dox fluorescence (red) (Fig 4 and higher magnification images in supporting information Fig 4).The increasing number of cytoplasmic nanoscale fluorescent burst release processes (represented in yellow), which is typically seen in cells with a more stretched morphology such as BLM and HUVEC, and the increasing nuclear Dox fluorescence (red) in most other cell types indicates that the heat-triggered Dox release continued to increase up to 1h.

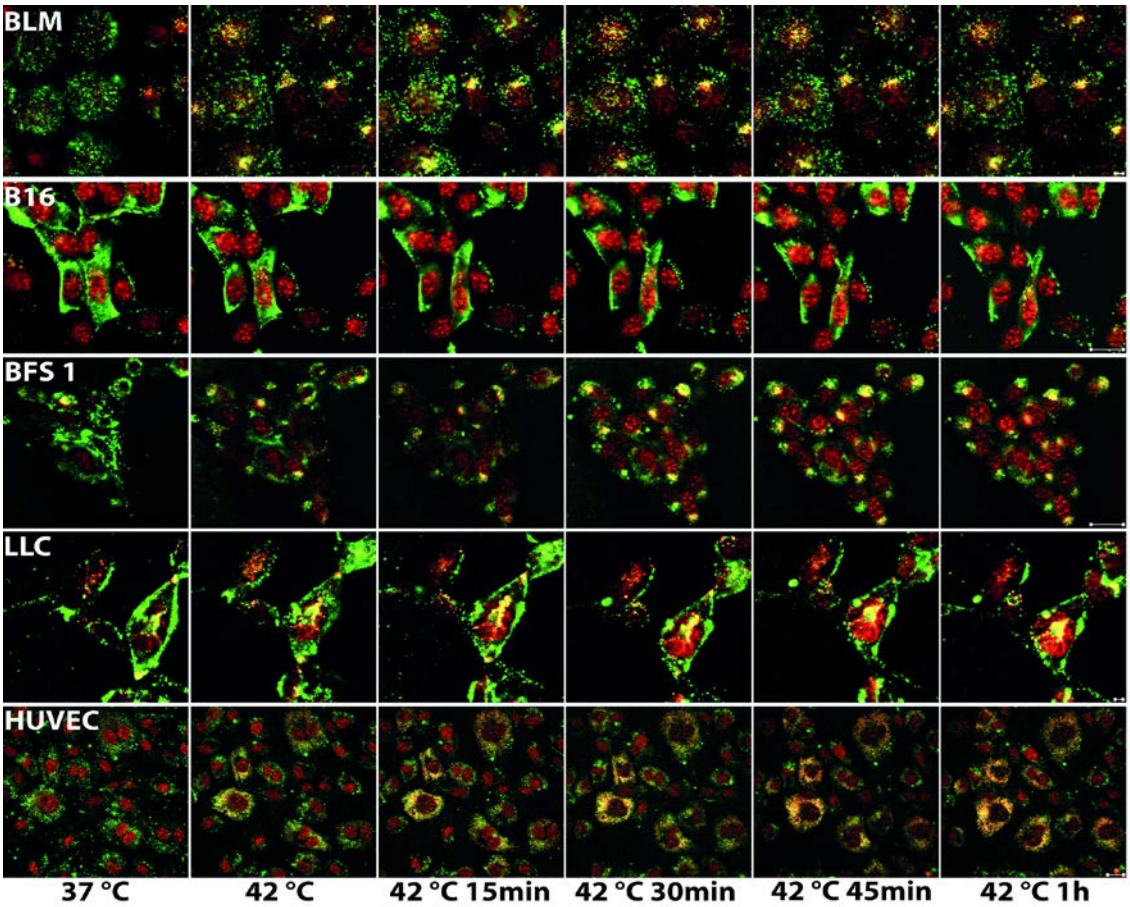

Fig 4. Doxorubicin release (red) from NBD-PE labelled CTSL (green) in BLM, B16, BFS1, LLC and HUVEC upon heat. Cells were incubated with Dox-CTSL (50 µM) for 1h at 37 °C, after which the cells were washed 3x with medium without FCS. Dox release was triggered at 42 °C for 1h. Images were taken by confocal microscope. Scale bar applies for all images, 20 µm.

3.7. Binding to and extravasation from tumor vasculature of TSL and CTSL

To evaluate the in vivo potential of the CTSL targeted and triggered Dox delivery approach, intravital confocal microscopic imaging in B16-tumor bearing mice expressing eNOStag-GFP (green) in their endothelial cells was performed. Using this advanced optical imaging technology we studied the intratumoral fate of CTSL, in vivo triggered Dox release during 1h of HT and the intratumoral fate of Dox. Dox-CTSL or TSL carrying a fluorescent bilayer probe DiD (purple) were administered i.v. and allowed to circulate for 300 min to study CTSL tumor targeting. During this normothermic targeting phase tumor vascular levels of TSL decreased due to nanoparticle clearance (Fig 5B left panel and C). By contrast, total CTSL fluorescence in vasculature remained relatively constant due to CTSL (purple) binding to tumor vasculature (green) represented as immobile fluorescence concentrated in patchy spots associated with endothelial cells, which increased over time (Fig 5A left panel) Next to bound CTSL, circulating CTSL levels (mobile fluorescence) decreased due to clearance and tumor vasculature binding (Fig 5B and C). This targeting event was not observed with TSL, which fluorescence appeared homogeneously distributed and mobile in the lumen of tumor vessels (Fig 5B left panel). Quantification of DiD-labelled liposomes in multiple images from ≥ 3 mice per treatment group showed that both TSL and CTSL fluorescence decreased similarly slow with time, representing comparable clearance from circulation in the period up to 300 min after injection (Fig 5C). This is remarkable, in view of the charge differences between both types of liposomes which did not seem to affect clearance. A HT treatment of 60 min at 42 °C following the targeting phase induced extravasation of both CTSL and TSL represented as diffused extravascular DiD fluorescence (fig 5A and B right panels marked with white arrows). Extravasation, which can promote nanoparticle delivery in the tumor interstitial space and subsequent binding to tumor cells started about 30 min after HT was initiated and increased in time up to 60 min confirming earlier observations with non-targeted TSL [27]. Extravasation of CTSL during 60 min of HT at 42 °C was confirmed using Rho-PE labelled CTSL (supplemental Fig 6). However, extravasation of both DiD and Rho-labelled liposomes was heterogeneous throughout the tumor and was not observed in every position and to the same extent. Remarkably, HT seemed to further promote binding of CTSL to tumor vasculature represented by increasing DiD fluorescence colocalizing with tumor vessels in the merged images, confirming earlier findings. [20] Interestingly, quantification of the liposomal DiD-fluorescence in intravital images indicates that the decline in intratumoral liposome fluorescence observed during the 5h targeting phase is halted and increases during hyperthermia. This increase may represent enhanced liposome extravasation of both formulations and increased CTSL binding.

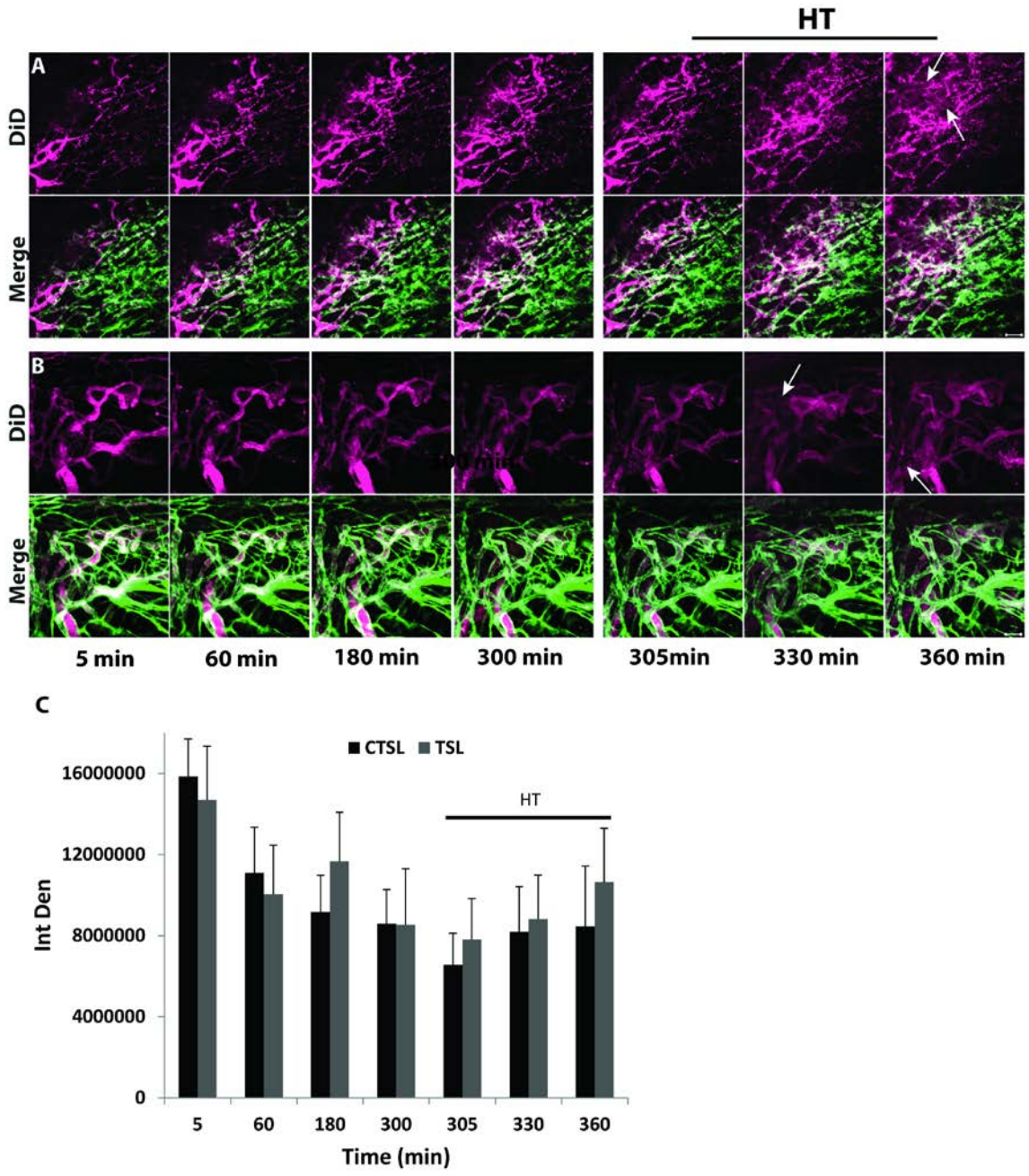

Fig 5. CTSL (A) and TSL (B) appearance (DiD, in purple) in tumor vasculature (green) during 300 min at NT in B16 window bearing mice (Fig 6 and B left panels) and upon subsequent HT at 42 °C for 60 min (Fig 6A and B right panels). DiD-labelled CTSL or TSL were injected through the penile vein, after which they were allowed to circulate in blood stream at NT for 300 min in order to allow binding of CTSL to angiogenic endothelial cells. Thereafter, HT at 42 °C for 60 min was applied to promote

extravasation of CTSL (A) and TSL (B). Scale bar applies to all images 50 µm. C. In vivo quantification of DiD liposomal fluorescence before and during 60 min of HT, presented as integrated density (IntDen) in time, see materials and methods for details.

3.8. In vivo Dox release, Dox uptake by tumor and endothelial cells and vessel destruction from Dox-CTSL

During 5h of circulation of Dox-TSL and Dox-CTSL, Dox tumor accumulation was low, and seemed slightly higher for TSL than CTSL, indicating some premature Dox release during the targeting phase from both formulations (Supplemental Fig 5). A HT of 42 °C applied at this 5 h time point however, triggered massive Dox release from both CTSL and TSL (Fig 6A). Dox release started when tissue temperature reached 42 °C and continued to increase with time. CTSL delivered higher levels of Dox to the tumor compared to TSL. Quantification of Dox fluorescence from the images demonstrated 2.7 fold higher Dox fluorescence delivered to the tumor by CTSL than by TSL (Fig 6B). Remarkably, Dox fluorescence appearance from TSL seemed to arise mainly from the vasculature (Fig 6A lower panel), whereas CTSL triggered Dox release appeared more homogeneous. High magnification intravital imaging demonstrated tumor vasculature (green) bound CTSL (purple) and Dox (red) delivered to endothelial cell and tumor cell nuclei (Fig 6C and supplementary video 1). CTSL delivered Dox was massively taken up by endothelial cells and tumor cells surrounding the blood vessels 24h after injection (Fig 6D). CTSL induced massive tumor vessel damage, which was heterogeneous in the tumor (Fig 6D). In contrast, no tumor vasculature damage was observed when TSL were applied (supporting information, Fig 7).

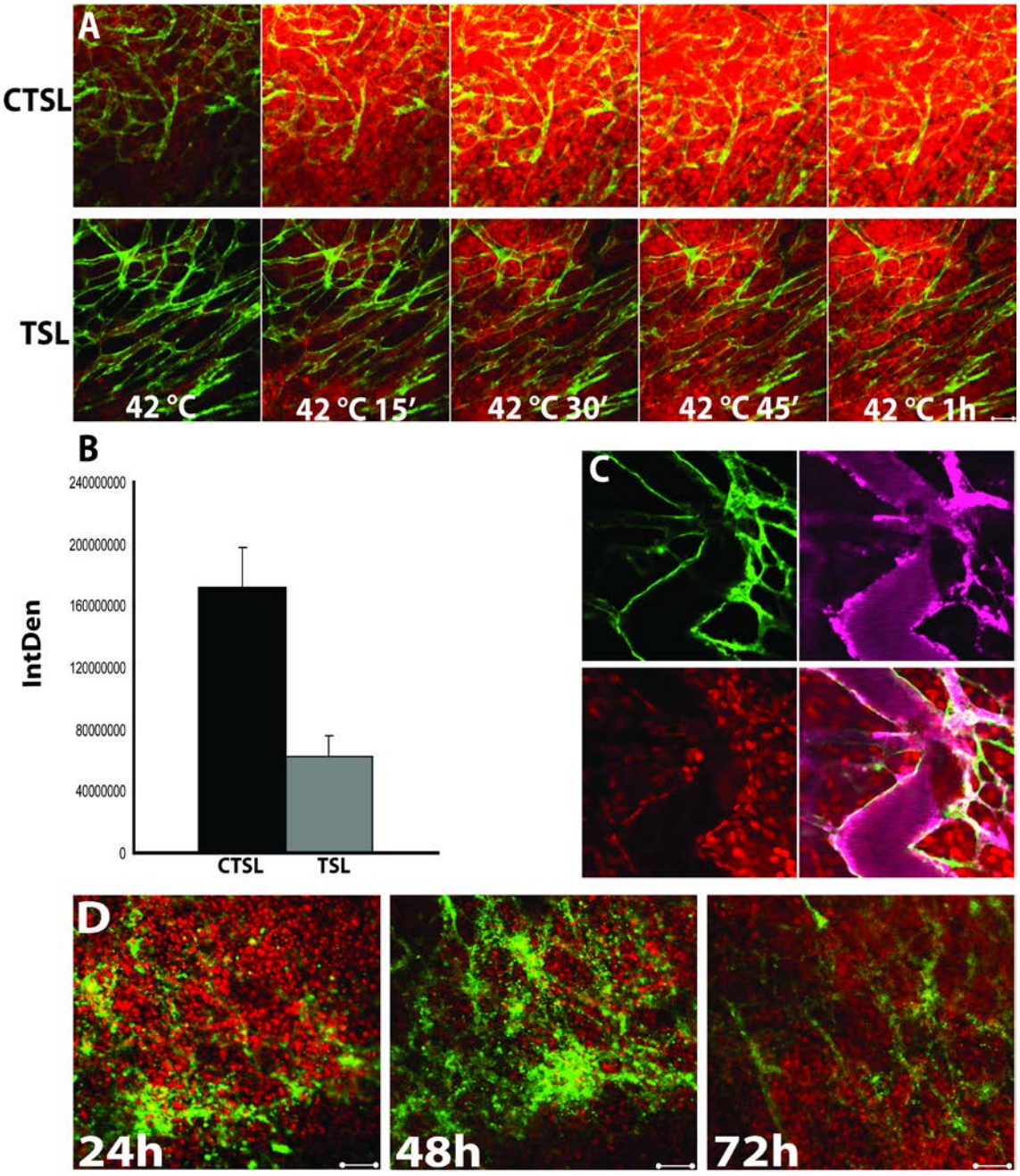

Fig 6. Dox release upon HT from CTSL (A upper panel) and TSL (A lower panel) in B16 window bearing mice. Temperature of 42 °C for 1 h was applied to the mice injected 5 h earlier with either Dox-CTSL or Dox-TSL. The i.v. injected Dox concentration was 5mg/kg. Images from mice injected with either Dox-CTSL or Dox-TSL were taken during 1 h of HT treatment. Scale bar applies for all images, 50 µm. B. In vivo quantification of Dox released from CTSL or TSL 1 h after HT treatment,

presented as integrated density (IntDen), see materials and methods. C. DiD labelled Dox-CTSL (purple) binding to and nuclear Dox uptake (red) by angiogenic endothelial cells (green) and tumor cells in a B16 tumor model. D. destruction of tumor vasculature at 24, 48 and 72 h after treatment with Dox-CTSL and HT. Scale bar 20 μm.

Discussion.

Various types of liposomal nanoparticles encapsulating chemotherapeutic drugs have been successfully developed and are being used in the treatment of cancer. [37] Although nanoparticle encapsulation can diminish drug side effects tremendously, their limited tumor accumulation [38], and intrinsic stability [9-11], hinder to increase therapeutic efficacy. [6, 8] Designing thermosensitive liposomes aids to control drug release locally in the tumor. [32, 33] The application of HT to thermosensitive liposomes allows their increased extravasation and accumulation in the tumor area. [22, 26, 27, 39] Dox encapsulated in lysolipid-thermosensitive liposomes (Dox-LTSL) has been developed for an intravascular drug release approach [40] and is used currently in clinical trials. [41] An alternative way of treating cancer with TSL involves an interstitial drug release, in which liposome extravasation from the tumor vasculature is promoted first. [42] Next to controlling drug release through an external heat trigger, cell-specific targeting of the nanoparticles may help to further improve therapeutic outcome. Recently we reported on combining the targeting and triggered release functions in one nanocarrier and developed CTSL, a triggered release device targeting both tumor and angiogenic endothelial cells. [20] In the present study we redesigned CTSL to accommodate the chemotherapeutic drug Dox for preferential targeting to angiogenic endothelial and tumor cells, to deliver high quantity of drug to tumors in an externally controlled, triggered manner.

CTSL design was modified to create a pharmaceutically stable formulation upon Dox loading. A slight decrease in the amount of cationic lipid content of the CTSL bilayer to 7.5 mol % was required to achieve a homogeneous and stable formulation regarding size, pdI, encapsulation efficiency and drug release, while still retaining its cell-binding capacity through a positive surface charge. The resulting Dox-CTSL formulation showed drug release kinetics at various temperatures similar to TSL lacking the cationic lipids, which were proven successful in vitro and in vivo at 42 °C in a recent study. [23] Dox-CTSL demonstrated stability up to temperature of 39 °C and rapidly released the encapsulated drug at 42 °C showing almost 100% release in 10 min (Fig 1), characteristics favourable for use in clinical settings. Fluorescence microscopy confirmed stability of TSL and CTSL at physiological temperatures (37 °C) for 1h, showing only minimal intracellular drug delivery (Fig 2). In contrast, HT at 42 °C triggered massive intracellular drug release in several tumor cell types and primary cultured endothelial cells being more abundant for CTSL than TSL, which is in accordance with the increased binding of CTSL to tumor and angiogenic endothelial cells. [20] The increased Dox delivery to all the cell lines by CTSL compared to TSL induced by the targeting function, was confirmed in a quantitative fluorometric assay (Fig 2). The induced specific cellular targeting and uptake of CTSL compared to TSL

leading to enhanced drug delivery resulted in increased cytotoxicity (Table 2). A HT trigger could not further increase the cytotoxicity of Dox-CTSL in any of the cell lines. This is likely due to the nature of the assay in which cytotoxicity is measured 72 h after nanoparticle incubation and heat treatment. During the remaining 72 h also non-heat triggered CTSL will release their Dox contents intracellularly due to cellular nanoparticle processing, causing cellular cytotoxicity. A possibly contributing factor may relate to the intracellular localization of CTSL in acidic compartments in the cytosol. Co-staining of acidic organelles (lysosomes) demonstrated that despite not all liposomes were yet associated with lysosomes, an important part of them did end up in this acidic cell compartment (Fig 4 and supporting information, Fig 3). This may hinder release of Dox, which in protonated form has more difficulty to pass membrane barriers [43], preventing its rapid nuclear accumulation. It may be interesting to further promote Dox release from acidic cell compartments by virtue of pH-sensitive functions that affect endo-lysosomal membrane stability at low pH. This approach caused faster nuclear Dox accumulation compared to non-pH sensitive liposomes [44-46]. Triggered intracellular Dox release in vitro from CTSL entrapped in cytoplasmic vesicles was visualized by live cell confocal microscopy as intracytoplasmic fluorescent nanobursts (Fig 4 and supporting information, Fig 4). This HT-triggered drug release resulted in an increased nuclear Dox uptake in studied tumor cell lines, but was less clear for endothelial cells, which showed a high number of cytoplasmic Dox nanobursts. This is likely due to slower endo-lysosomal Dox release in endothelial cells compared to tumor cells. [43]

During a 5 h circulation of CTSL in the blood stream (targeting phase) minimal Dox accumulation in tumor was observed suggesting minimal premature Dox release (Supporting information, Fig 5), which correlates nicely to their stability in vitro when exposed to 37 °C in serum. (table 1). Targeting of Dox-CTSL to tumor vasculature was demonstrated during the 5 h targeting phase (Fig 5) confirming that adaption of particle design and Dox loading did not affect the targeting potential of CTSL that we observed earlier [20]. Application of HT at 42 °C for 1h seemed to further promote the binding of Dox-CTSL to tumor vasculature, which may be due to increased negatively charged molecules on the endothelial cell membranes. In accordance with Li et al [27], HT also induced extravasation of CTSL and TSL, which appeared heterogeneous within the tumor area. Extravasation started around 30 min after application of HT and increased up to 1h (Fig 5). Remarkably, quantification of liposomal fluorescence at various positions in the tumor in multiple animals indicated that CTSL were not cleared faster from circulation than TSL (Fig 5C). Interestingly, the levels of liposomal fluorescence of both CTSL and TSL increased upon HT for 1h, which may represent enhanced extravasation as well as possible binding of CTSL. Importantly HT triggered abundant Dox release especially from CTSL and to a lesser extent from TSL (Fig 6). Quantification of numerous position in the tumors showed that Dox-CTSL delivered nearly 3-fold higher Dox quantities than TSL. This indicates that due to the positive charge Dox-CTSL have improved capacity to increase Dox delivery to tumors compared to TSL. Since both formulations had similarly low levels of serum induced

Dox leakage (Table 1), increased intratumoral retention and cellular uptake by tumor and endothelial cells (Fig 2 and Fig 6C) are likely responsible for the enhanced drug delivery using Dox-CTSL. Specificity of cationic liposomes for angiogenic endothelial cells has been previously reported by several groups. [47] Campbell et al [17] have shown in their study in a DSC mouse model that only 4% of the injected cationic liposomes associated with the normal vasculature whereas 25-28% were delivered to tumor vessels. In addition, the specific binding of cationic liposomes to tumor vasculature appeared not dependent on tumor type. They speculated that the reason for this preferential delivery was the sluggish and irregular blood flow in tumor vasculature that facilitated the binding between positively charged cationic liposomes and anionic sites on tumor vasculature. A study by Thurston et al [14] showed that angiogenic blood vessels in RIP-Tag2 and K14-HPV16 mice took up 15-33 fold more cationic liposomes than normal vessels. Additionally, this study showed that angiogenic endothelial cells in tumors and chronically inflamed tracheas took up cationic liposome-DNA complexes, but not anionic, neutral or sterically stabilized neutral liposomes. In accordance with our findings and Thurston et al [14], Krasnici et al [18] also proved the specific uptake of cationic liposomes by tumor endothelium compared to anionic and neutral liposomes in a melanoma window chamber model in Syrian hamsters. Finally, a study by Dellian et al [48] in ortotopically grown pancreatic cancer revealed that in both early and late tumorigenesis cationic liposomes bound preferentially to tumor microvessels compared to normal pancreatic microvessels. Moreover, the same study showed that the delivery of fluorescent paclitaxel-containing cationic liposomes was increased in tumors compared to normal surrounding tissue. In our previous study [49] in window chamber bearing mice implanted with either melanoma B16 or Lewis lung carcinoma tumor models, we have shown that cationic thermosensitive liposomes bound specifically to tumor endothelial cells even 20 min after injection. By contrast, in neither of the tumor models neutral thermosensitive liposomes were found associated with angiogenic endothelial cells even at later time points.

HT-triggered drug release resulted in massive drug uptake by both tumor and angiogenic endothelial cells, confirming the dual targeting approach. Additionally, only CTSL were able to cause massive tumor vasculature destruction, most probably due to their vascular targeting and drug delivery potential, which is in accordance with studies using non-triggerable cationic formulations by Abu-Lila et al. [19] using oxaliplatin loaded cationic liposomes and Kunstfeld et al [50] using paclitaxel loaded cationic liposomes.

Conclusion.

CTSL were successfully redesigned to accommodate Dox as a chemotherapeutic drug with remaining dual targeting and heat-triggered release functions. The novel Dox-CTSL complied with all requirements for in vivo application, i.e. a homogeneous and stable nanoparticle formulation with targeting specificity towards angiogenic endothelial and tumor cells. With these advanced properties Dox-CTSL promoted delivery of high Dox quantities intracellularly into various tumor cell lines and endothelial cells in vitro and to tumors in vivo. HT-induced intracellular Dox nanoburst

events demonstrated that temperature triggered drug release within the cell is a feasible option. Massive CTSL association with tumor vasculature and hyperthermia promoted CTSL extravasation and tumor cell targeting delivered high quantities of Dox into the tumor. Next to direct tumor cell kill the observed tumor vessel damage can further contribute to an antitumor effect. Further studies will address the efficacy of our novel dual targeted and triggered doxorubicin delivery approach using Dox-CTSL.

Acknowledgements

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Supplemental information

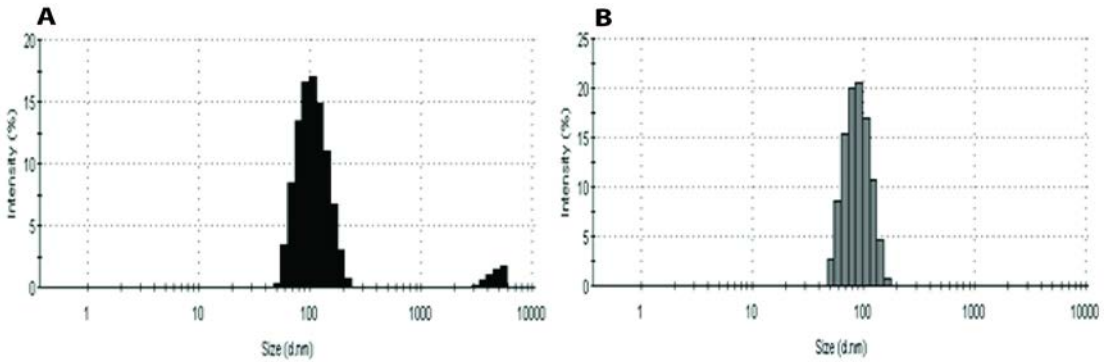

Fig 1. Size distribution of Dox-CTSL-10 (A) and Dox-CTSL-7.5 (B). Decreasing the amount of the cationic lipid resulted in a more homogeneous liposome formulation.

Table 1. Characterization of empty TSL, CTSL-7.5 and CTSL-10. Data are represented as mean ± standard error of the mean (SEM), N=4.

Batch	Size (nm)	PdI	Zeta potential (mV)	T_m (°C)
TSL	84±0.7	0.05±0.0	-9.9±0.3	44.3*
CTSL-10	89±1.3	0.06±0.0	11.9±0.5	45.6
CTSL-7.5	84±2.9	0.04±0.0	6.1±1.1	45.4

*Dicheva BM, 2013. [20]

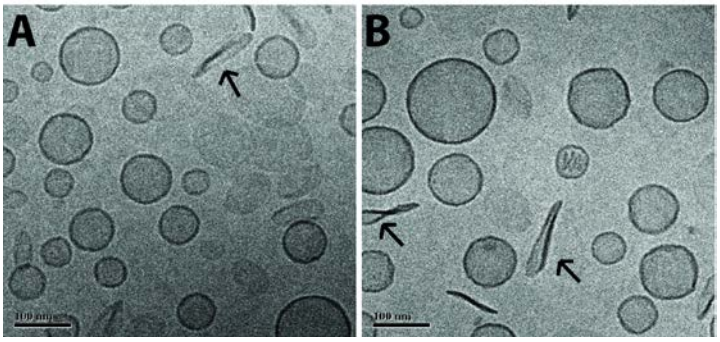

Fig 2. TEM cryo imaging of empty CTSL-7.5 (A) and CTSL-10 (B). CTSL formulations consisted of liposomes, and disc shaped micelles (DSMs) which were observedin the images of both formulations (black arrows), but more abundant for CTSL-10. Bar, 100nm.

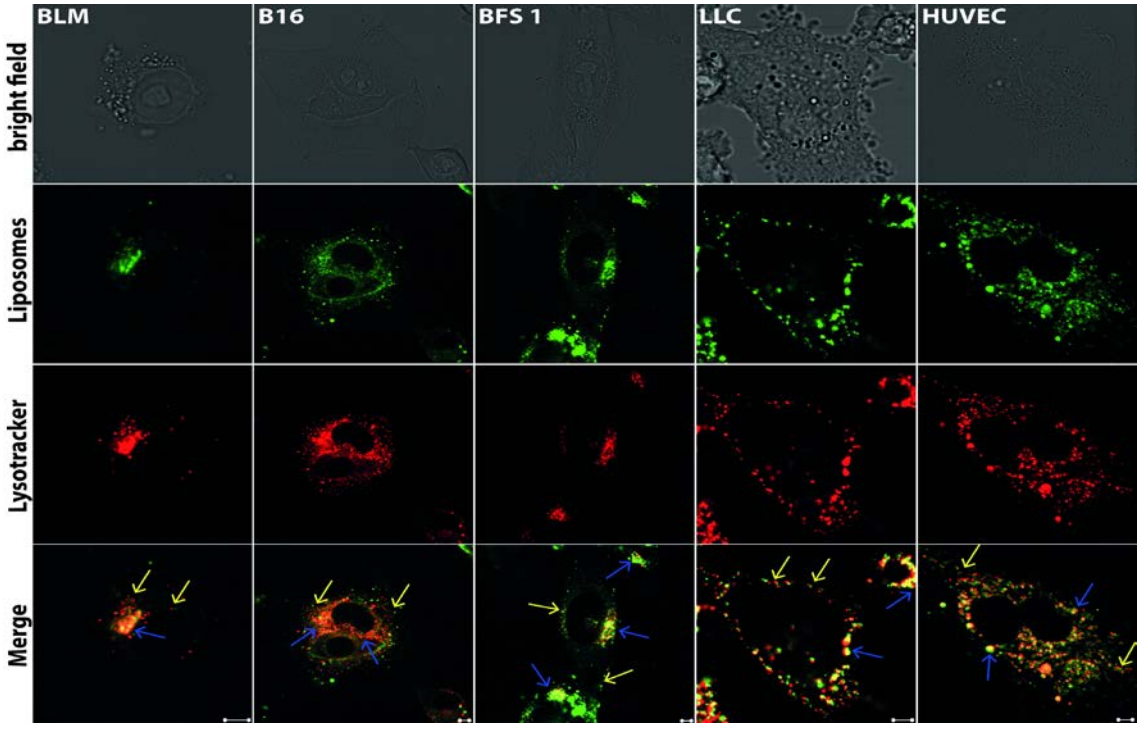

Fig 3. Colocalization of CTSL with lysosomes 1h after incubation and removal of unbound liposomes. Liposomes (green) were observed colocalized (yellow) with the lysotracker (red). Colocalization is indicated with blue arrows. Liposomes which were not colocalized with lysotracker were also observed in all cell lines (yellow arrows). Images were taken with a confocal microscope (40x/NA1.30, pinhole 2,5 µm). Scale bar applies to all images, 20 µm.

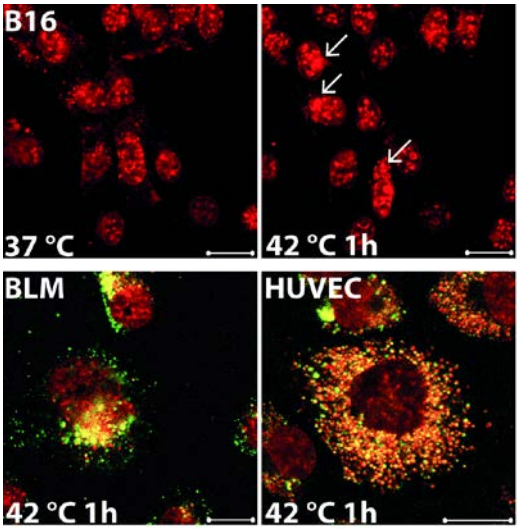

Fig 4. Release of Dox (red) upon HT at 42 °C for 1h in B16, BLM and HUVEC. The application of HT for 1h caused additional Dox release (red) in the cytoplasm of B16 (Top panels, marked with white arrows). Magnifications of BLM and HUVEC treated with Dox-CTSL and HT clearly show released Dox in the cytoplasm from internalized liposomes (green), seen as yellow colocalization. Images were taken with a confocal microscope. Scale bar applies to all images 20 μm.

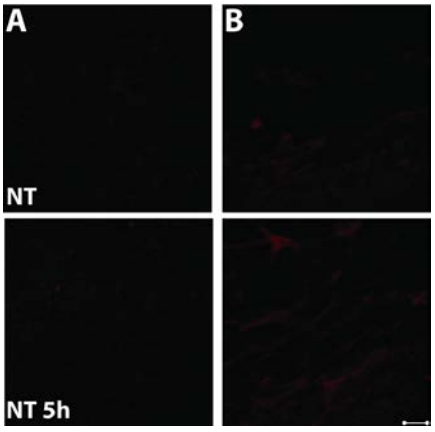

Fig 5. Stability of Dox-CTSL (A) and Dox-TSL (B) in circulation. Liposomes were injected i.v. in penile vein of B16 window bearing mice at 5 mg/kg Dox, after which they were allowed to circulate for 5h. Images were taken right after injection at NT and 5h later in order to observe possible accumulation of prematurely released Dox in the tumor. Scale bar applies for all images, 50 μm.

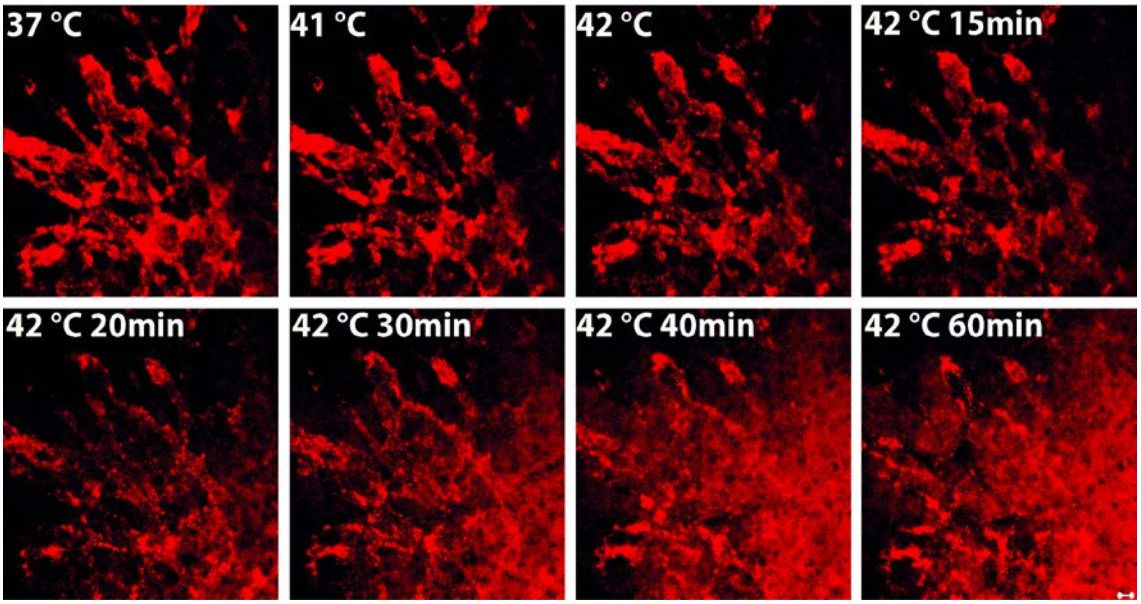

Fig 6. Extravasation of Rho-PE labelled CTSL (red) upon heat. CTSL (5 µmol) were injected into B16-tumor bearing mice and allowed to circulate for 2h. After 2h, hyperthermia at 42 °C for 1h was applied. Hyperthermia caused massive liposome extravasation observed by diffusely distributed red fluorescence outside blood vessels and the presence of less liposomes inside the blood vessel lumen. Scale bar applies to all images 20 µm.

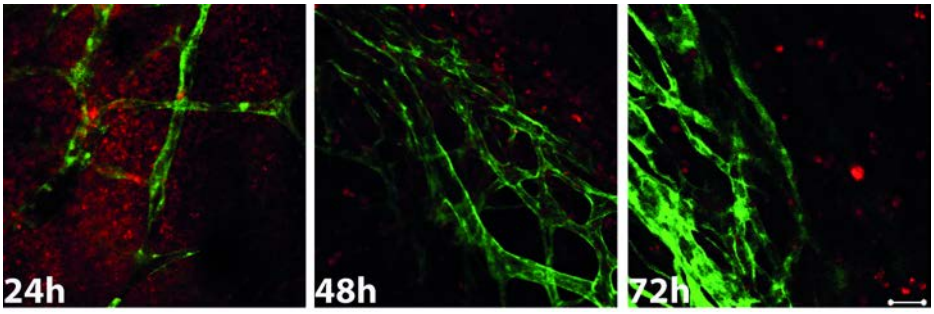

Fig 7. Absence of tumor vasculature damage when B16-tumor bearing mice were treated with Dox-TSL in combination with 1h of HT. Representative images were taken for different time points (24, 48 and 72h) after the HT treatment. Dox (red) can be observed outside blood vessels (green) and is cleared from the tumor in time. Scale bar applies for all images 50µm.

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Chapter 4

Enhanced specificity and drug delivery in tumors by cRGD - anchoring thermosensitive liposomes

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Abstract

Purpose: To develop RGD-targeted thermosensitive liposomes with increased tumor retention, improving drug release efficiency upon mild hyperthermia (HT) in both tumor and angiogenic endothelial cells.

Methods: Standard termosensitive liposomes (TSL) and TSL containing a cyclic Arg-Gly-Asp (cRGD) pentapeptide with the sequence Arg-Cys-D-Phe-Asp-Gly (RGDf[N-Met]C) were synthetized, loaded with Dox and characterized. Temperature- and time-dependent drug release profiles were assessed by fluorometry. Intracellular Dox delivery was studied by flow cytometry and confocal microscopy. Cytotoxic effect of TSL and RGD-TSL was studied on B16BL6 melanoma, B16F10 melanoma and HUVEC. Intravital microscopy was performed on B16BL6 tumors implanted in dorsal-skin fold window-bearing mice. Pharmacokinetic and biodistribution of Dox-TSL and Dox-RGD-TSL were followed in B16BL6 tumor bearing mice upon normothermia or initial hyperthermia conditions.

Results: DLS and cryo-TEM revealed particle homogeneity and size of around 85 nm. Doxorubicin loading efficiency was >95% as assessed by spectrofluorometry. Flow cytometry and confocal microscopy showed a specific uptake of RGD-TSL by melanoma and endothelial cells when compared to TSL and an increased doxorubicin delivery. High resolution intravital microscopy demonstrated specific accumulation of RGD-TSL to the tumor vasculature. Moreover, application of hyperthermia resulted in massive drug release from RGD-TSL. Biodistribution studies showed that initial hyperthermia increases Dox uptake in tumors from TSL and RGD-TSL.

Conclusion: RGD-TSL have potency to increase drug efficacy due to higher uptake by tumor and angiogenic endothelial cells in combination with heat-triggered drug release.

Keywords: doxorubicin, RGD, thermosensitive liposomes, hyperthermia, drug delivery

Introduction.

Nanoparticles, such as liposomes have passed different stages of modifications in their design and nowadays are commonly used in cancer chemotherapy [1]. Stealth liposomal nanoparticles of 100 nm are believed to accumulate passively in the tumor due to the leaky tumor vasculature and related enhanced permeability and retention effect [2, 3]. Liposomes have contributed significantly to decrease toxic side effects caused by free drug administration [4-6]. However, tumor accumulation of anticancer drugs using liposomes seemed far from optimal to guarantee improvement in therapeutic efficacy in clinical practice [4, 7]. Low specificity of liposomes and their high intrinsic stability limit therapeutic outcome. Selectivity and efficacy can be achieved by decorating liposomes with targeting ligands, while an external trigger, e.g. heat, can control liposomal drug release. In this study we aimed at developing RGD-targeted thermosentitive liposomes, which combine active targeting of tumors together with a heat-triggered drug release function. These nanoparticles contain multiple RGD

peptides on their surface to achieve tumor specificity and increased retention in tumors and a thermosensitive bilayer for heat-triggered drug release.

In order to efficiently target tumor cells, liposomes first need to extravasate from tumor vasculature and penetrate the tumor tissue. However, the extravasation process is usually heterogeneous and inefficient [3, 8]. Targeting of tumor vasculature rather than tumor cells has become a promising approach in cancer therapy [9]. In this case several tumor pathophysiological barriers do not play a role as endothelial cells are easily accessible to circulating chemotherapeutic drugs and deep penetration into tumor tissue is not necessary. Moreover, destruction of tumor vessels leads to indirect killing of tumor cells, which depend on their supply of oxygen and nutrients. Finally, endothelial cells are genetically stable and therefore less resistant to drug therapy [10]. Besides vascular targeting, those small liposomes may extravasate through the leaky tumor vasculature and target tumor cells in addition [11].

Various vascular targets have been studied for anti-vascular therapy of which αvβ3 integrins have been used most often [12, 13]. These integrins have been found to be overexpressed on tumor vasculature, but also on some metastatic melanoma cells [14, 15]. The RGD (Arg-Gly-Asp) sequence is known to be a recognition motif for integrins such as αvβ3 [16]. Binding to either tumor or endothelial cells via specific receptors may lead to internalization of liposomal chemotherapy thereby bringing the drug closer to the nucleus. The combination of targeting properties of this nanoparticle with the heat-triggered release function might aid at releasing the drug locally in the tumor. Besides triggering drug release, hyperthermia (HT) is known to play a role in changing tumor environment by increasing tumor blood flow, oxygenation and vessel permeability [16-21].

An abundant amount of literature is available on thermosensitive or targeted liposomes [22-25]. However, the combination of both strategies in one carrier is a promising approach. In the present study we describe the design, characterization and behavior of RGD-targeted thermosensitive liposomes containing the chemotherapeutic drug doxorubicin in vitro and in vivo. These liposomes were decorated with a novel and specific cyclic Arg-Gly-Asp (cRGD) pentapeptide containing the sequence Arg-Cys-D-Phe-Asp-Gly (RGDf[N-Met]C) [26]. We tested the cytotoxic effect of those liposomes on melanoma cell lines and endothelial cells. Extensive live cell imaging was performed to study their intracellular fate in vitro. In vivo, their affinity for tumors, drug release kinetics and uptake were studied in dorsal skin fold window chamber models implanted with melanoma B16Bl6 tumors. Pharmacokinetics and biodistribution of Dox encapsulated in either TSL or RGD-TSL were investigated in mice implanted with B16Bl6 tumors.

Materials and methods.

Chemicals. The phospholipids 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-distearoyl-sn-glycero-3-

phosphoethanolamine-N-PEG2000 (DSPE-PEG2000) were ordered from Lipoid (Ludwigshafen, Germany). 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3 benzoxadiazol-4-yl) (NBD-PE) and 1,2-Disteroyl-sn-Glycero-3-Phosphoethanolamine-N-[Maleimide (Polyethylene Glycol)2000] (Ammonium Salt) were purchased from Avanti Polar Lipid Inc. Doxorubicin-HCl was from Pharmachemie (Haarlem, The Netherlands). The RGDf[N-Met]Cys was provided by Peptron, South Korea. Sodium 3'-[(1-phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) was purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). LysoTracker Red DND-99 and Dioctadecyl tetramethylindotricarbocyanine perchlorate (DiD-C_{18}(3)) were purchased from Invitrogen.

Preparation of TSL. TSL and RGD-TSL were composed of DPPC:DSPC:DSPE-PEG_{2000} in a molar ratio 70:25:5. RGD peptide was coupled to mPEG in a molar ratio 1.1:1 (peptide:lipid) and coupling efficiency was confirmed by MALDI analysis. For MALDI analysis, all peptide samples were prepared as 0.001 M in water. The matrix-assisted laser desorption/ionization (MALDI) 2,5-dihyfroxy benzoic acid was prepared as 10 mg/mL in water. 10 uL of sample and 90 uL of matrix solution were mixed and 0.5 uL of this mixture was spotted on an anchor chip plate and allowed to dry at ambient temperatures. MALDI-TOF mass spectra were recorded on a Bruker Ultraflex III mass spectrometer, Bremen, German. [27] Coupling of >95% was used for further liposome preparation. Liposomes were prepared by lipid film hydration and extrusion method [16]. The lipids were dissolved in chlorophorm and methanol (9:1 vol/vol). TSL used for confocal microscopy contained 0.3 mol% of NBD-PE or 0.3 mol% DiD in the lipid bilayer. TSL used for intravital microscopy contained 0.3 mol% of DiD. The solvent was subsequently evaporated in a rotary evaporator until homogeneous lipid film was formed. The lipid film was hydrated in 250 mM $(NH_4)_2SO_4$ solution with a pH 5,0 at 60 °C for 30 min. The spontaneously formed liposomes were extruded subsequently 5 times through 100 nm, 5 times through 80 nm and 5 times through 50 nm polycarbonate filter (thermo barrel extruder at 60 °C) and resulted in < 100 nm TSL. $(NH_4)_2SO_4$ outside of liposomes was removed from liposomal $(NH_4)_2SO_4$ by a PD-10 Sephadex column (GE Healthcare, Buckinghamshire, UK), eluted with HEPES buffer, pH 7.4 (10 mM HEPES, 135 mM NaCl). Size and polydispersity index (PDI) were measured by dynamic light scattering using Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Lipid concentration was analysed by phosphate assay [27]. After the phosphate concentration was determined, doxorubicin was loaded into the liposomes in 0.05:1 drug:lipid ratio (mol:mol) at 38 °C for 1h. The liposomes were concentrated by ultracentrifugation for 2h, 4°C, 106.000 xg (Ti50.2 rotor). The pellet was resuspended in HEPES buffer, pH 7.4 and left overnight on slow rotation at 4 °C. Then the liposomes were passed through PD 10 column eluted with HEPES buffer, pH 7.4 to remove free doxorubicin. Doxorubicin concentration was measured by spectrophotometer at Ex 480 nm and loading efficiency (%) determined as [Dox/Lipid] after loading/[Dox/Lipid] before loading x100.

TEM cryo imaging. Samples were prepared by adding a 2 µl droplet of liposome suspension to a lacy carbon film and subsequently plunge-freezing this sample into liquid ethane using a Vitrobot. An amorphous ('vitrified') ice film which contains the particles of interest was created. Cryo-TEM studies were performed using a FEI TECNAI F30ST (300kV, using a cryo-holder, keeping the sample at –174 ºC during the studies). Imaging was done in low-dose mode on a CCD camera (image size 1k x 1k).

Stability at physiological conditions. Stability of TSL and RGD-TSL was established by incubating 10mM [lipid]) in pre-heated FCS (1:149 v/v) under stirring and Dox release was measured for 1h at 37 °C. Samples without incubation were considered as a blank (Io). TSL were destroyed by adding 10% Triton X-100 (150:1 v/v) and considered as a positive control (I_{∞}). Fluorescence was measured by fluorometry at Ex. 479 nm / Em. 590 nm (Hitachi F-4500 Fluorescence Spectrophotometer). Dox release was determined as Dox (%) = (It−Io) / (I_{∞}−Io) × 100. Stability of liposomes was calculated as 100 – Dox (%).

In vitro Dox release. Temperature-dependent Dox release kinetics from TSL and RGD-TSL were performed by fluorometry upon incubating the TSL samples (10mM [lipid]) in pre-heated 90% fetal calf serum (FCS) (1:9 v/v) at temperatures ranging between 37-45 °C for 5 min in a thermal-shaker (Eppendorf Thermomixer) at 300 rpm. Samples without incubation were considered as a blank (Io). After incubation, the samples were diluted in 10 mM Tris/NaCl 0.9%, pH 8.0 at 1:50 (v/v) and Dox fluorescence was measured by fluorimetry at Ex. 479 nm / Em. 590 nm. Maximum Dox fluorescence (positive control) (I_{∞}) was achieved when incubating TSL suspension (10mM [lipid]) in 2% Triton X-100 in H_2O for 30 min in a thermal shaker at 55 °C and 1400 rpm. The Dox release (%) was calculated as Dox (%) = (It−Io) / (I_{∞}−Io) × 100. In vitro time-dependent Dox release from TSL and RGD-TSL was measured at 42 °C. TSL suspension (10 mM [lipid]) was mixed with pre-heated FCS (1:149 v/v) under stirring and Dox release was measured over time (at 1, 2, 3, 4, 5, 30, 60 min). TSL samples without heating were considered as a blank. TSL were destroyed by adding 10% Triton X-100 (150:1 v/v) and considered as a positive control. The Dox release (%) was calculated as described above.

Cell culture. Tumor cell lines B16Bl6 (murine melanoma) and B16F10 (metastatic murine melanoma) were cultured in a Dulbecco's Modified Eagles' medium (Lonza, Belgium) containing 10% FCS. Human umbilical vein endothelial cells (HUVECs) were isolated in-house and cultured in Human Endothelial SFM medium (Gibco, Invitrogen) enriched with 30% FCS. Cells were passaged once a week using Trypsin (Sigma, Aldrich) and maintained at 37 °C, 5% CO_2 in a humidified incubator. All experiments were performed at a confluence of 80-90%.

In vitro Dox-TSL toxicity. B16Bl6, B16F10 and HUVEC cells were plated in 96 well plates at concentration 12000 cells/well for B16Bl6 and B16F10 and 6000/well for HUVEC. The cells were allowed to attach for 24h at 37 °C and after that incubated with

various concentrations of free Dox, Dox-TSL or RGD-Dox-TSL for 3h at 37 °C. After 3h, liposomes were removed and cells washed 3 times with medium with FCS. Plates were placed at either normothermia (NT; 37 °C) or hyperthermia (HT; water bath at 42 °C for 1h) and then left in the incubator at 37 °C for additional 72h. Cell survival was determined by XTT assay. Electron coupling reagent *N*-methyl dibenzopyrazine methylsulfate (1.25 mM in PBS; Sigma) (100 µl) was mixed with 5ml of XTT solution (1 mg/ml in RPMI 1640) and each well was incubated with 50µl of this mixture for 1h at 37 °C. Afterwards, XTT conversion was measured at 490 nm in a PerkinElmer Victor Wallac plate reader (Perkin Elmer, Groningen, The Netherlands).

Flow cytometry. Binding of TSL or RGD-TSL to B16Bl6, B16F10 and HUVEC cells was assessed by flow cytometry (FACS) analysis. $1x10^5$ cells in suspension were incubated for 3h at 37 °C with 400 nmol/ml TSL or RGD-TSL labelled with 0.3 mol% NBD-PE. After incubation, cells were washed three times with medium with FCS to remove unbound liposomes. Liposomal NBD-PE fluorescence was determined at excitation and emission wavelengths of 470 and 530 nm by a BD FACScan (Becton Dickinson, San Jose, CA, USA). Dead cells were labelled with propidiumiodide (PI) (Sigma, Aldrich) and ten thousand gated events were acquired per sample and samples were prepared in triplicate. For Dox uptake FACS analysis, the same amount of cells was incubated with 100 µM Dox for 3h and subsequently washed 3x with medium with FCS. Cells in suspension were placed either at normothermia (37 °C) or hyperthermia (42 °C) for 1h. Dox fluorescence was determined at excitation and emission wavelengths of 488nm and 585nm, respectively. As a dead cell marker, Sytox Green (Invitrogen) was used. Data was analysed with FlowJo software. Experiments were performed three times with three different batches of liposomes.

Live cell confocal microscopy. B16Bl6, B16F10 or HUVEC cells were seeded at the same concentrations as for fluorescent microscopy in cell culture chambers containing a cover glass insert coated with 0.1% gelatine. Cells were allowed to recover for 24 hours. After 24h, cells were incubated with 400 nmol/ml NBD-PE labelled TSL or RGD-TSL for 3h at 37 °C and for 30 min with lysotracker (LysoTracker® Red DND-99). After incubation, cells were washed 3 times with DMEM (B16Bl6 and B16F10) or HUVEC medium (HUVEC). Cells were analyzed on a Zeiss LSM 510 META confocal laser scanning microscope. NBD-PE fluorescence was detected by 513 nm argon laser and lysotracker was monitored by a 543 nm Helium –Neon laser. For Dox release experiments, cells were incubated with NBD-PE or DiD labelled Dox-TSL or RGD-Dox-TSL for 3h at 37 °C and after that washed 3 times with medium with FCS. Dox release was followed in time for 1h at 42 °C (40 x objective lens, 2.5 µm pinhole*)* and its fluorescence was detected by a 543 nm Helium –Neon laser. Images of 1024 x 1024 pixels were analyzed using Zeiss LSM image software (Zeiss, Germany).

Animal models. B16Bl6 (murine melanoma) cells were cultured in DMEM medium with 10% FCS. Ten million tumor cells were injected subcutaneously in the flanks of

C57Bl6 mice and bulk tumors of 10 mm in diameter were used for transplantation into C57Bl6, expressing an eNOS-tag-GFP fusion protein constitutively in their vascular endothelium. Tumor pieces were implanted in a dorsal skin flap window chamber for intravital imaging. Bulk mice were housed at 20-22 °C, humidity of 50-60%. Window chamber-bearing mice were used for experiments after 8-12 days of tumor implantation when tumor size reached 4-6 mm in diameter. These mice were housed in an incubator room with a humidity of 70% and temperature of 30-32 °C. All mice were fed a standard laboratory diet *ad libitum* (Hope Farms Woerden, the Netherlands). Mice weighing 20-25 g were used for experiments. All animal experiments were performed in compliance with protocols approved by the committee on Animal Research of the Erasmus MC, Rotterdam, The Netherlands.

Intravital microscopy for Dox release upon hyperthermia and uptake by tumor and angiogenic endothelial cells. Liposome binding to tumor vasculature and their clearance from circulation was analysed by intravital microscopy after injection of DiD-labelled TSL or RGD-TSL and followed up to 24h or 5h respectively. In order to evaluate Dox release during hyperthermia and its uptake by tumor vascular endothelial cells and tumor cells, DiD-labelled Dox-TSL or RGD-Dox-TSL were injected i.v. through the penile or tail vein at a dose of 5mg/kg Dox. Both formulations were allowed to circulate for 5h at body temperature in order to be able to bind to vascular angiogenic endothelial cells or tumor cells and observed by confocal microscopy (Zeiss LSM 510 META). After 5h of circulation, tumor was heated at 42 °C for 1h and Dox release and uptake was detected as above (20x objective lens). Regions of interest were selected before, during and in the end of the hyperthermia treatment. Images of 1024 x 1024 pixels were analyzed using Zeiss LSM image software (Zeiss, Germany).

In vivo dox quantification. The integrated density (IntDen) from the red channel (obtained after setting a threshold) representing released doxorubicin at 42 °C into mice injected with either RGD-Dox-TSL or Dox-TSL was quantified from 13 positions from each group, which were obtained from 3 mice. The data are presented as an average of IntDen of all the positions of each mouse. The data was analyzed by Image J software. When DiD quantified, 3-6 positions were used per mouse obtained from 3 mice from each group. The data are presented as an average of IntDen of all the positions of each mouse.

Pharmacokinetic and biodistribution of Dox-TSL and Dox-RGD-TSL. Pharmacokinetic and biodistribution of Dox-TSL and Dox-RGD-TSL was followed in B16Bl6 tumor bearing mice upon NT or initial HT conditions. At NT condition, mice were injected with 3mg/kg Dox and blood sampling was performed at 0.1; 1; 2; 4; 6 and 24h and organs were collected 24h after liposome injection. At HT condition, tumors were first preheated for 1h at 41 °C and then cooled down for 15 min, in order to facilitate liposome extravasation. Then, liposomes were injected at 3mg/kg Dox and blood samples were collected up to 24h (0.1; 1; 2; 6; 24h), after which the organs were

removed. The Dox concentration in the blood and organs was analyzed by HPLC and calculated as % injected dose/g tissue (%ID/g). 6 mice were used per each group.

Statistics. *In vivo* drug release was analyzed by Mann-Whitney test and results with p-value ≤ 0.05 were considered statistically significant.

Results.

Liposome characterization

RGD-TSL and TSL consisted of the phospholipids DPPC, DSPC, DSPE-PEG2000 in a molar ratio (70:25:5). In the RGD-TSL formulation, RGD peptide was coupled to $mPEG_{2000}$ and its coupling efficiency was analyzed by MALDI (Fig 1), after which it was mixed with all the lipids in a molar ratio (70:25:5). Both formulations were prepared by lipid film hydration and extrusion method [28]. Dox was loaded into the liposomes after extrusion by $(NH_4)_2SO_4$ loading method [29]. Liposomes were characterized by measuring size, polydisperity index (pdI), encapsulation efficiency and stability. All liposomes were ~85 nm after extrusion and with a pdI < 0.1. After Dox loading, liposomes retained their small size and a low pdI. Encapsulation efficiency of both formulations was >95%. Liposomes were comparably stable after 1h of incubation at 37 °C in 99% FCS. TSL contained 92% ± 1.8 of the entrapped Dox after 1h at 37 °C whereas RGD-TSL contained 85% ± 2.3 (data not shown).

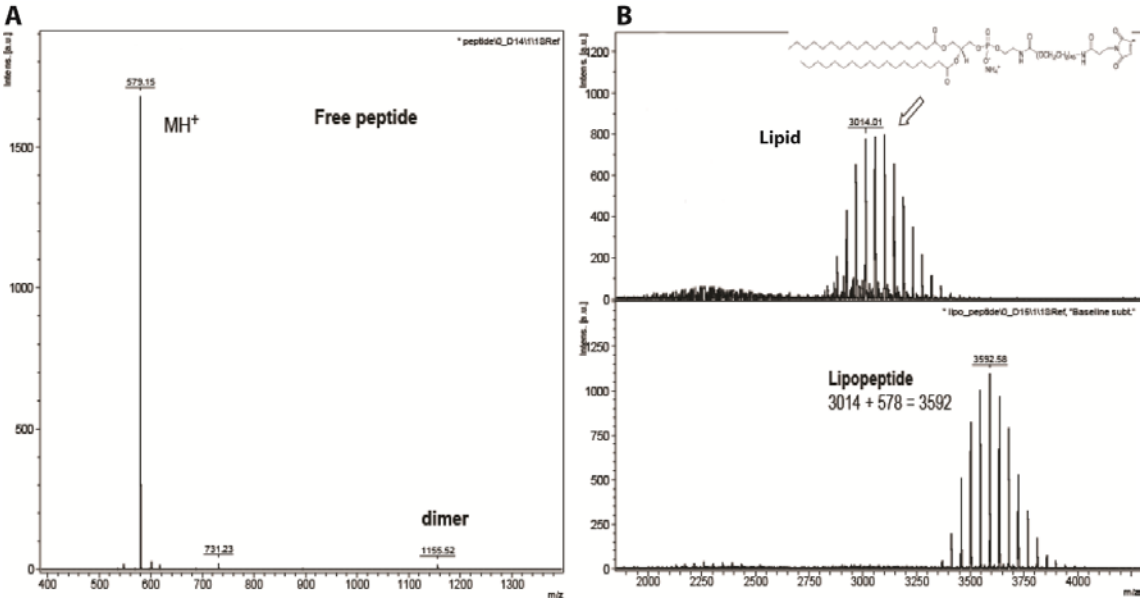

Fig 1. MALDI-TOFF spectra of the free peptide RGD (A), the free lipid (B upper panel) and the coupled RGD to mPEG in lipopeptide (B lower panel).

Temperature- and Time-dependent Dox release

In order to test whether liposomes are thermosensitive, their temperature- and time-dependent drug release profiles were determined (Fig 2A and B). According to the temperature-dependent release kinetics (A), which was performed at varying temperatures (37-45 °C), both formulations were thermosensitive showing an increasing drug release with increasing temperature. The formulations were stable at physiological temperatures (37-38 °C) in 5 min and started to release their drug payload slightly at 39 °C. The maximum drug release from TSL in 5 min was observed at 42 °C (~85%), whereas for RGD-TSL it was at 43-44 °C (~80%), after which temperatures the drug release declined as seen before [21].

Time-dependent release profile at a constant temperature of 42 °C for 1 h was similar for both formulations (Fig 2B). RGD-TSL and TSL rapidly released Dox in the first minute and released >90% of the encapsulated Dox in 1h.

Active loading of Dox into RGD-TSL and TSL by ammonium sulphate gradient method resulted in an abundant amount of intraliposomal Dox crystals, which were well visualized by TEM cryo imaging in both formulations (Fig 2C).

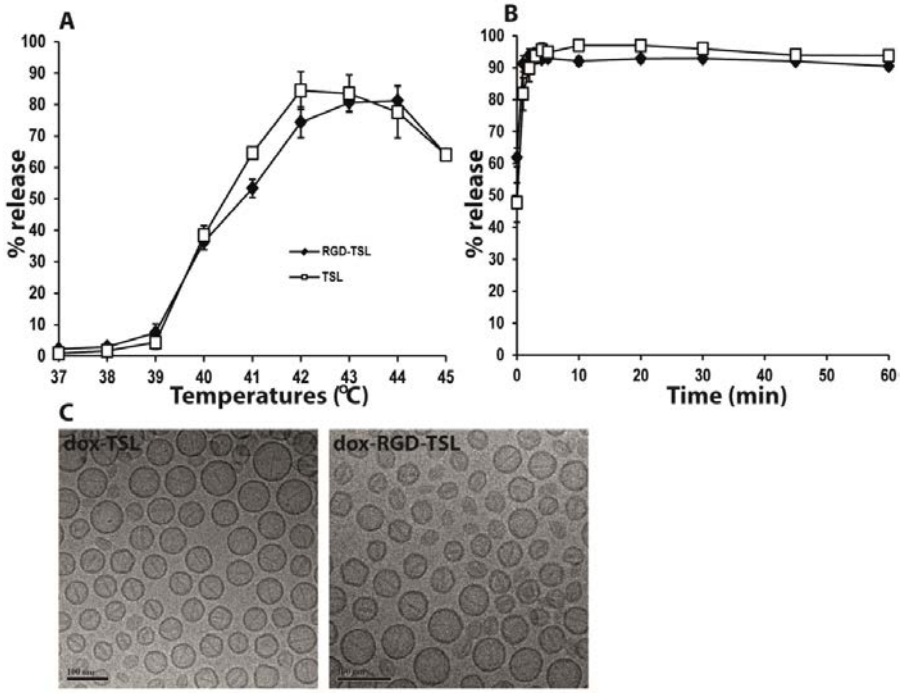

Fig 2. Temperature- (A) and time- (B) dependent Dox release kinetics from RGD-TSL and TSL. Temperature-dependent drug release profile was performed in temperatures between 37-45 °C for 5 min in 90 % FCS. Time-dependent release was carried for 1 h at 42 °C in 99.7 % FCS. Mean of three independent experiments with three different batches of liposomes. TEM-cryo imaging (C) of Dox-TSL and Dox-RGD-TSL. Bar, 100nm.

RGD-TSL demonstrate higher uptake by tumor and endothelial cells than non-targeted TSL

In order to test whether RGD coupling to liposomes increases liposomal uptake into tumor and endothelial cells, confocal microscopy and FACS analysis on melanoma B16Bl6 and B16F10 cells and HUVEC with NBD-PE labelled TSL or RGD-TSL was performed. Confocal microscopy on B16Bl6, B16F10 and HUVEC demonstrated that RGD-TSL are more abundantly taken up by the tumor cells and HUVEC compared to TSL (Fig 3A). The 3h incubation period at 37 °C followed by a washing step for removal of unbound liposomes revealed that the RGD modification of TSL led to a preferential binding and uptake by all the cell lines. This was confirmed also by FACS analysis (Fig 3B). In order to measure Dox delivery, FACS analysis was performed in B16Bl6, B16F10 and HUVEC after 1h (C) or 3h (D) of incubation with Dox-TSL or Dox-RGD-TSL followed by 1h of HT at 42 °C. The amount of Dox delivered from RGD-TSL at NT was higher than TSL in all cell lines and this amount further increased after 3h of incubation. When HT was applied to the cells after 1h of incubation at 37 °C, there was an increase in Dox release and uptake in all cell lines. HT trigger did not further increase the delivered Dox to B16Bl6 and B16F10 cells after 3h of incubation. Only in HUVECs there was an increased delivery of Dox upon HT after 3h of incubation.

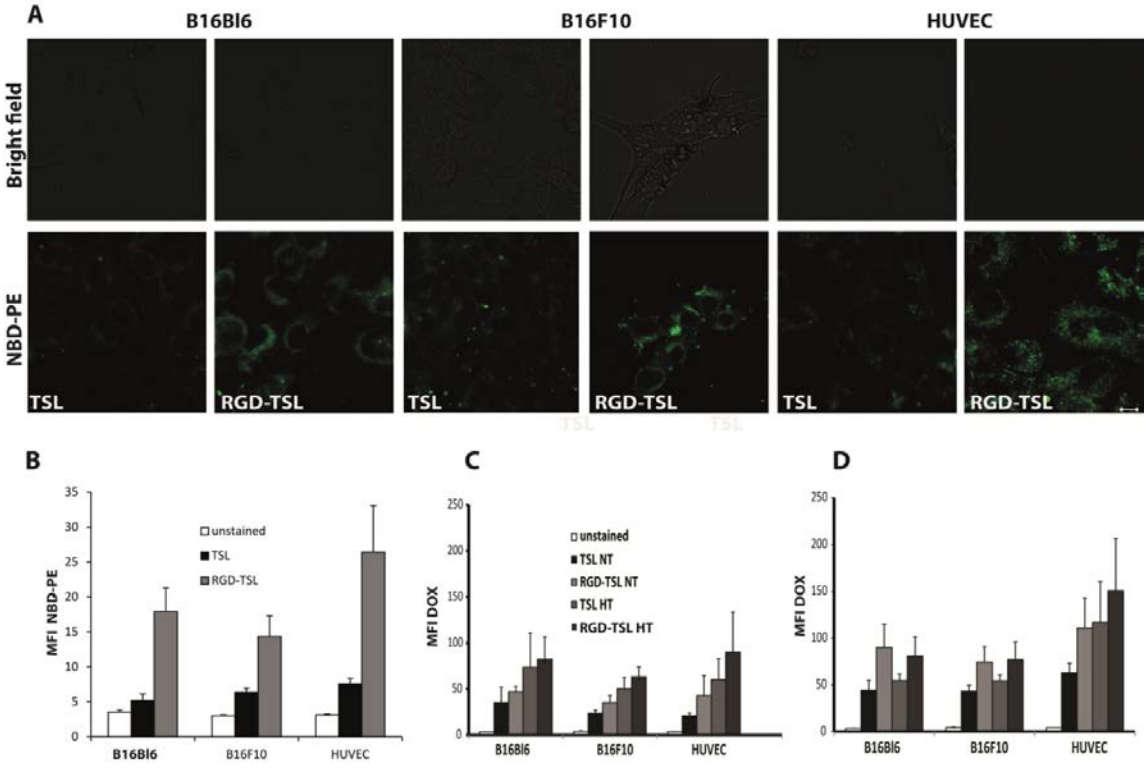

Fig 3. Confocal live-cell imaging for preferential uptake of RGD-TSL compared to TSL into B16Bl6, B16F10 and HUVEC cells after 3h of incubation at 37 °C (A). Unbound liposomes were removed by washing. Scale bar applies for all images, 10µm. Intracellular NBD-PE fluorescent intensity represented as mean fluorescent intensity (MFI) in melanoma B16Bl6, B16F10 cells and HUVEC (B) treated with either TSL or RGD-TSL for 3h at 37 °C. Unbound liposomes were removed by washing. As unstained cells are used cells which were not incubated with liposomes. C and D. Intracellular Dox uptake represented as MFI in melanoma B16Bl6, B16F10 cells and HUVEC after 1h (C) or 3h (D) of incubation at 37 °C, washing of unbound liposomes, followed by 1h of HT at 42 °C.

Uptake of RGD-TSL in lysosomes and intracellular, HT-triggered Dox release from RGD-TSL in tumor and endothelial cells

The intracellular localization of liposomes was studied by live cell imaging in B16Bl6, B16F10 and HUVEC (Fig 4A). Cells were incubated with NBD-PE labelled (green) RGD-TSL and lysotracker (red, to stain lysosomes) for 3h at 37 °C, after which the unbound liposomes were removed by washing. B16Bl6 and B16F10 were able to localize the RGD-TSL in the cytoplasm after 3h of incubation and those liposomes were colocalized with the red lysotracker, seen as yellow fluorescence signal (white arrows). Still, there were some RGD-TSL (green fluorescence spots), which were not concentrated in the acidic compartments (blue arrows). By contrast, in HUVEC sequestering of RGD-TSL in the lysosomes occurred at a slower pace. After 3h of incubation at 37 °C, liposomes and lysotracker were observed as green and red separated fluorescence signals, showing no entrapment of the liposomes in the lysosomes. However, when cells were followed for a prolonged period, colocalization (in yellow) started to be visible. After 7h of incubation, there was an abundant amount of RGD-TSL localized in the lysosomes but also some non-entrapped liposomes in lysosomes could be observed.

To prove that Dox-RGD-TSL deliver the encapsulated drug intracellular, live cell imaging on B16Bl6, B16F10 and HUVEC was performed using DiD labelled Dox-RGD-TSL and green lysotracker (Fig 4B). Removing the unbound liposomes 3h after incubation at 37 °C resulted in some doxorubicin release inside of all cell lines, most probably due to processing of liposomes by the cells in this time frame. The released Dox localized mainly in the cell nuclei but could also be observed in the cytoplasm. The application of HT for 1h triggered additional Dox release, seen as increased red fluorescence signal intracellularly. In B16Bl6 and B16F10, the Dox delivery upon HT treatment was predominantly nuclear, whereas in HUVEC it was cytoplasmic (Fig 4B and C). In accordance with Fig 4A, DiD-labeled RGD-TSL (purple) also localized in lysosomes (green) to some extent. Non-colocalized liposomes with lysosomes could also be observed. Interestingly, the released cytoplasmic Dox upon HT colocalized with

the green lysotracker, which indicates Dox release from RGD-TSL also occurs in acidic compartments in the cytosol (Fig 4B and C).

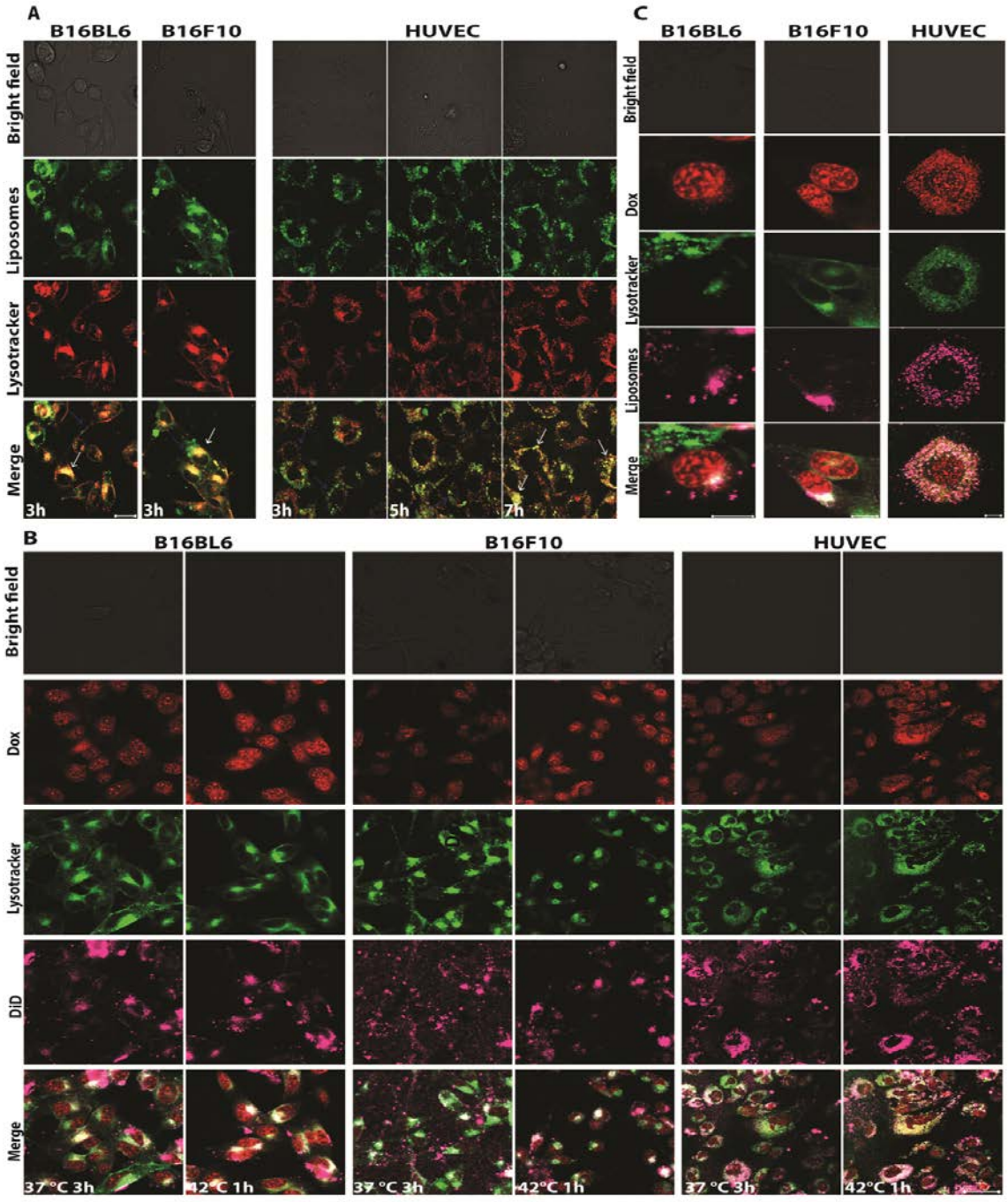

Fig 4. A. Confocal microscopy on melanoma B16Bl6 and B16F10 cells and HUVEC incubated with NBD-PE (green) labelled RGD-TSL for 3h at 37°C and lysotracker

(red). Unbound liposomes were removed by washing 3x with medium without FCS. After washing, B16Bl6 and B16F10 were immediately imaged at 37°C, whereas HUVEC were followed up to 7h. Internalized liposomes in the cytosol can be observed in all the cell lines (white arrows). Non-internalized in the lysosomes liposomes were also visible (blue arrows). B16Bl6 and B16 internalized liposomes immediately in the lysosomes after the 3h of incubation period (yellow colocalization of green liposomes and red lysotracker), whereas this process happened in HUVEC after 7h. Images were taken by confocal microscope (40x, 2.5µm pinhole, 2x zoom). Scale bar applies for all images, 20µm. B. Doxorubicin release (red) from DiD-labelled RGD-TSL (purple) in B16Bl6, B16F10 and HUVEC upon HT trigger. Cells were incubated with 50µM Dox for 3h at 37 °C, after which cells were washed 3x with medium without FCS. Images were taken right after this incubation at 37 °C. Then, HT at 42 °C for 1h was applied and images in the end of the HT treatment were recorded. Images were taken by confocal microscope. Scale bar applies for all images, 50µm. C. Colocalization of RGD-TSL (purple) with lysotracker (green) and Dox release (red) in the lysosomes. Scale bar applies for all images, 10µm.

Cytotoxicity of Dox encapsulated in TSL and RGD-TSL upon NT and HT

Incubation of B16Bl6,B16F10 and HUVEC cells for 3h with Dox-TSL or Dox-RGD-TSL showed no differences in cell toxicity determined 72h later as seen in Fig 5 and table 1. Application of HT for 1h at 42 °C after 3h of liposomes incubation could not further improve cytotoxicity impact 72h later in B16F10 and HUVEC but only to some extent in B16Bl6 cells. Free Dox demonstrated the highest cytotoxicity on all the cell lines because it is rapidly taken up by the cells in its free form.

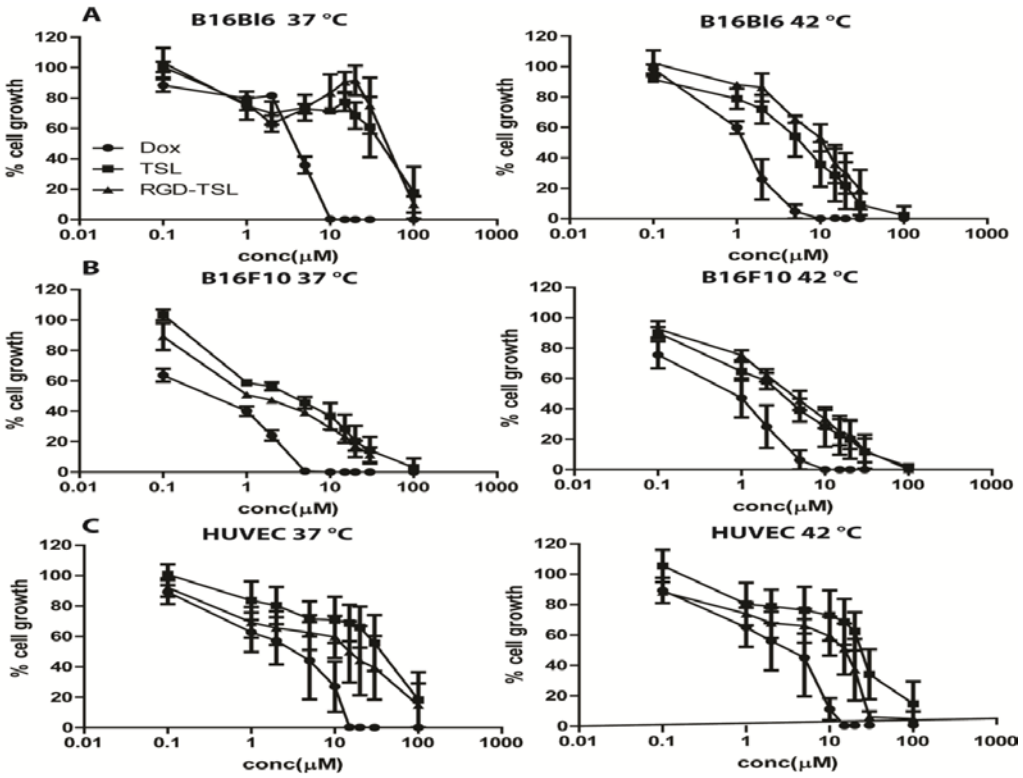

Fig 5. Cytotoxicity of Dox-TSL and Dox-RGD-TSL either at 37 °C or 42 °C in B16Bl6 (A), B16F10 (B) and HUVEC (C) determined 72h after liposome incubation.

Table 1. *IC 50 values in μM Dox of B16Bl6 murine melanoma, B16F10 murine melanoma and endothelial cell (HUVEC) treated with free Dox or Dox formulated in TSL or RGD-TSL for 1h at 37°C. After removal of unbound liposomes, cells were subjected to HT at 42 °C for another 1h (in blue) or NT (in black) as a control.

IC50 (μM)			
Treatment	B16BL6	B16F10	HUVEC
Dox	3.8±0.3	1.1±0.2	5.7±2.6
TSL	58.8±28	8.6±5.5	53.9±23.9
RGD-TSL	44.4±8.9	2.6±1.1	51.0±26.2
Dox	1.5±0.3	1.4±0.5	4.9±1.9
TSL	9.9±5.7	6.2±3.1	32.2±8.5
RGD-TSL	12.6±4.2	5.0±1.2	16.9±4.8

Binding to- and extravasation from tumor vasculature of TSL and RGD-TSL

In order to proof that RGD-TSL target angiogenic endothelial cells in vivo, intravital microscopy in B16Bl6 window chamber bearing mice was performed. To visualize circulating liposomes in the blood stream, liposomes were labelled with DiD (purple). In these mice tumor vasculature is visualized by the constitutive expression of a GFP-eNOS-tag fusion protein in endothelial cells (Fig 6). Twenty minutes after injection of RGD-TSL, next to circulating liposomes in the lumen of the blood vessels, bound RGD-TSL could be observed (Fig 6A yellow arrows). These liposomes can be visualized as patchy fluorescent spots on the vessel walls. Besides bound liposomes to the angiogenic endothelial cells, extravasated liposomes from tumor vasculature are visible already 20 min after injection (white arrows). They are visible as diffuse purple fluorescence outside of the green blood vessels. Binding continued in time and was pronounced 24h after injection. In contrast, using DiD-labeled TSL, no binding to tumor vasculature was detected after 5h of circulation. In the last time point (24h), only extravasated TSL were visible (Fig 6A, right panel).

Liposome clearance from circulation during the 5h targeting phase and Dox triggered release upon HT was further evaluated by intravital microscopy in B16Bl6 window chamber bearing mice (Fig 6B). Liposome clearance was observed already 2h after injection by decrease in DiD fluorescence signal from both RGD-TSL and TSL. Clearance of liposomes continued in time up to 5h (Fig 6B and C). However, RGD-TSL bound to tumor vasculature was visible already 5min after injection, whereas this was not observed for TSL even after 5h of circulation, which is in accordance with Fig 6A. HT trigger at 42°C for 1h did not seem to cause any additional clearance neither of RGD-TSL or TSL. Upon HT, TSL could be observed extravasated from the tumor vasculature (white arrows) a process which is known to be heterogeneous within the tumor. This is in accordance with the quantification of the images, showing an increase of the TSL signal upon HT, which is most probably due to extravasation. However, there was no increase of the RGD-TSL levels upon HT (Fig 6C).

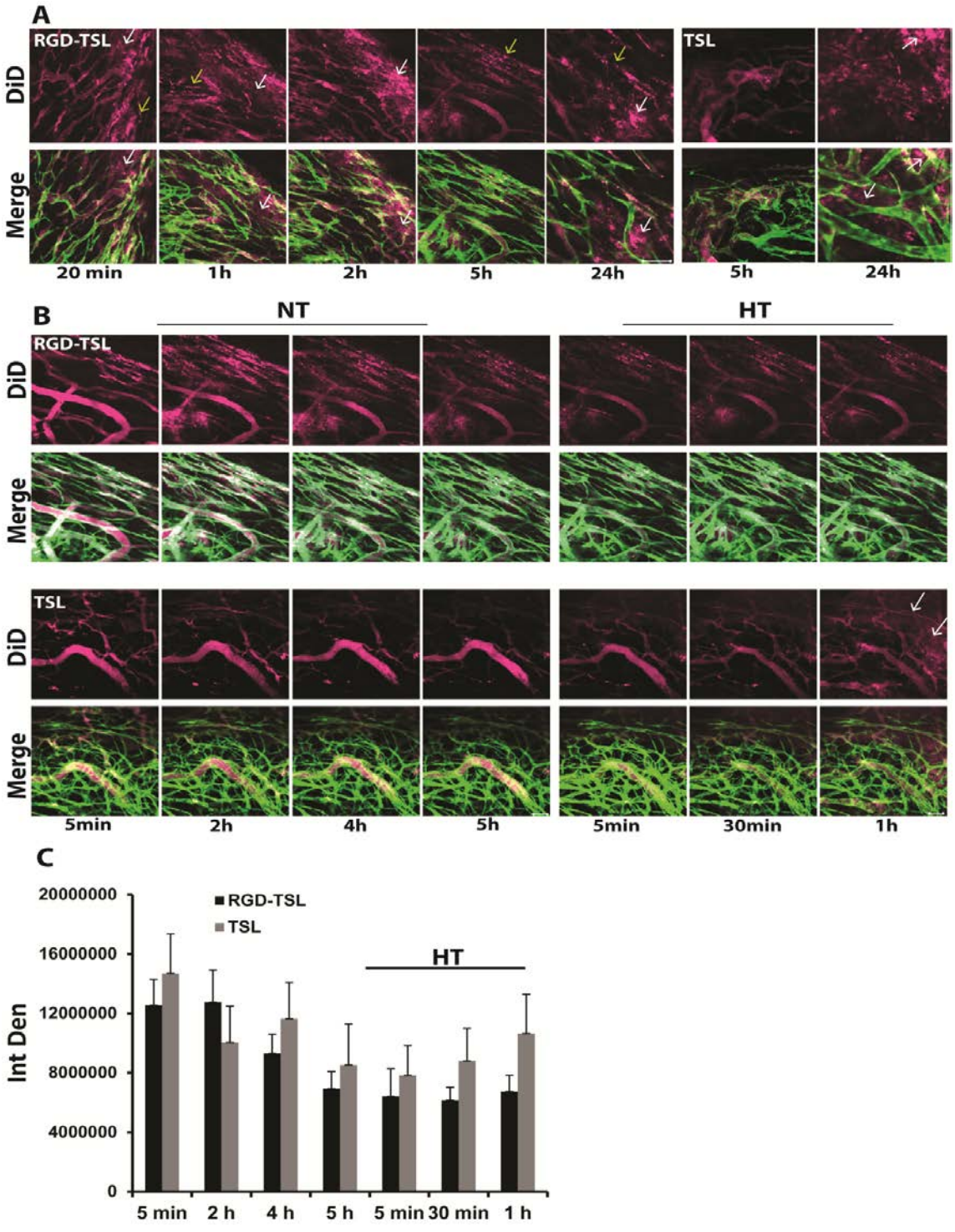

Fig 6. A. Binding of DiD-labeled RGD-TSL (purple) to tumor vasculature (green) of B16Bl6 window chamber bearing mice. Binding of liposomes to tumor endothelial cells

started 20min after injection and was followed in time up to 24h. Representative images from intravital microscopy were selected. Scale bar applies to all images, 50µm. B. RGD-TSL and TS appearance (DiD, in purple) in tumor vasculature (green) during 5h at NT in B16Bl6 window bearing mice (Fig B left panels) and upon subsequent HT at 42 °C for 1h (Fig 5B right panels). DiD-labelled RGD-TSL or TSL were injected i.v, after which they were allowed to circulate in blood stream at NT for 5h in order to allow binding of RGD-TSL to angiogenic endothelial cells. Thereafter, HT at 42 °C for 1h was applied to promote extravasation of RGD-TSL and TSL. Scale bar applies to all images 50µm. C. In vivo quantification of DiD liposomal fluorescence before and during 1h of HT, presented as integrated density (IntDen) in time, see materials and methods for details.

In vivo Dox release upon HT and Dox uptake by tumor and endothelial cells

In order to understand whether RGD-TSL release Dox in vivo upon HT treatment and to follow Dox distribution in the tumor, intravital microscopy was performed. Circulation of TSL and RGD-TSL for 5h in the blood stream did not cause any premature release of Dox (data not shown). However, when HT at 42 °C was applied, immediate Dox release (red) was observed from TSL (Fig 7A) and RGD-TSL (Fig 7B). Dox from both formulations was released first intravascular from the circulating DiD-labelled liposomes (purple), after which it was gradually taken up by endothelial cells and tumor cells surrounding the blood vessels (Fig 7D and supporting information, video 1). The Dox uptake in the both treatment groups increased in time and was maximal after 1h of HT, when also the lumen of the blood vessels was cleared from Dox. Quantification of the images showed that the amount of delivered Dox to the tumor from RGD-TSL was 1.7 fold higher than from TSL (Fig 7C). However, this difference was not statistically significant (p-value = 0.8).

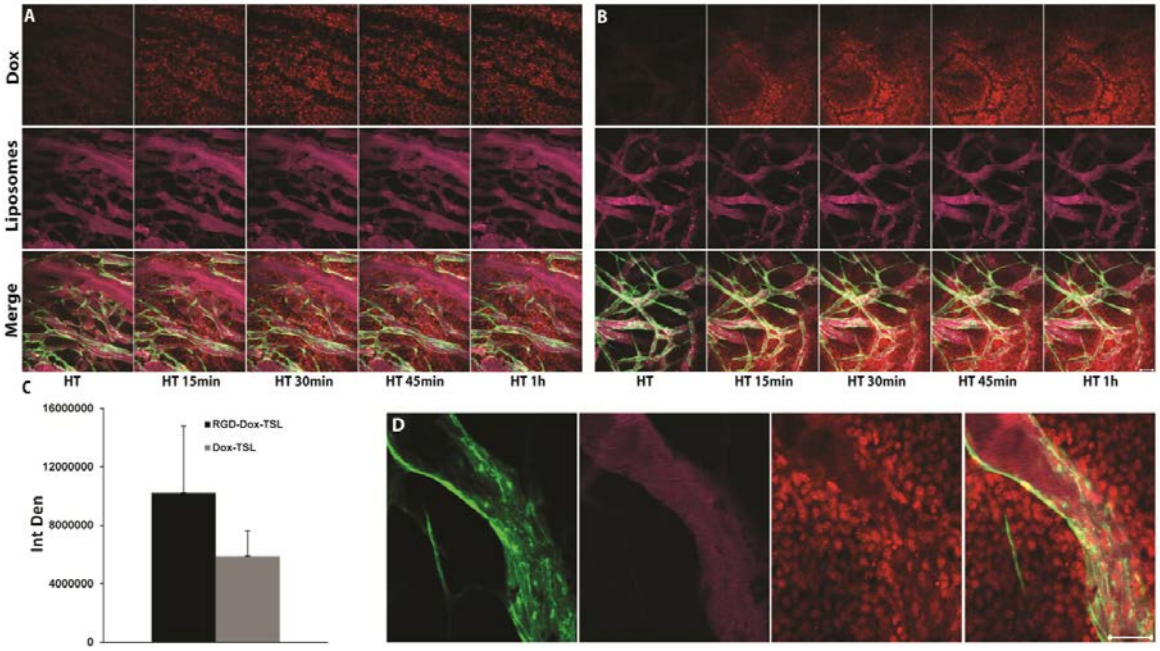

Fig 7. Dox release upon HT treatment from TSL (A) and RGD-TSL (B) in B16Bl6 window chamber bearing mice. Mice were injected with 5 mg/kg Dox in DiD-labelled (purple) TSL or RGD-TSL. After 5h of liposome circulation, a temperature of 42 °C for 1h was applied to trigger Dox release. Representative images were taken from the beginning of the HT treatment up to 1h. C. In vivo quantification of Dox released from RGD-TSL or TSL 1h after HT treatment, presented as integrated density (IntDen), see materials and methods. D. Dox uptake in endothelial cells (green) and tumor cells from RGD-TSL after 1h of HT treatment. Scale bar applies for all images, 50µm.

Pharmacokinetics and biodistribution of Dox-TSL and Dox-RGD-TSL

To follow up Dox clearance from circulation, its distribution in healthy organs and tumors and to be able to quantify Dox concentrations in tumors and organs, the pharmacokinetic and biodistribution profiles of Dox in TSL or RGD-TSL were studied (Fig 8) under NT or HT conditions. At NT condition (Fig 8A and C), Dox from both formulations seemed to clear from circulation quite fast in the first 1h, as it was faster for RGD-TSL than for TSL (27% v/s 52% remaining Dox respectively). After 2h of liposome circulation, the trend was the same showing lower remaining Dox from RGD-TSL than from TSL (10% v/s 20% respectively). At later time points (4, 6, 24h) there was barely any Dox present in circulation from TSL and RGD-TSL. The application of initial HT (Fig 8B) seemed to increase the presence of Dox from RGD-TSL in circulation at 1h time point, after which its clearance was the same as at NT conditions. Besides, upon initial HT conditions, clearance of Dox from TSL and RGD-TSL was similar. Considering the biodistribution of Dox (Fig 8C and D), at both NT and HT

conditions, there was a significant uptake of Dox from both formulations in the spleen as it was higher for Dox from RGD-TSL than TSL (11.3 respectively v/s 8.3% ID/g at NT; and 11 v/s 6 % ID/g at HT). Similar high Dox accumulation in the kidney was observed from both formulations. Dox accumulated in the liver was similar for RGD-TSL and TSL under NT (3.9 %ID/g respectively v/s 2.7 %ID/g). The higher Dox uptake in spleen and liver from RGD-TSL is due to most probably opsonization of RGD-TSL by proteins in these organs. There was a minimal uptake of Dox from TSL and RGD-TSL in the heart, lungs and muscle upon NT and HT. No Dox was detected in the brain from neither of the formulations. At NT, the tumor uptake of Dox was similar for both formulations. Application of initial HT for 1h at 41 °C was able to cause ~ 3.7 fold increase of Dox delivery to the tumor from RGD-TSL (1.6 v/s 6 % ID/g) and ~ 2.3 fold increased Dox amount to the tumor from TSL (1.7 v/s 4 % ID/g). The amount of Dox delivered to the tumor upon initial HT conditions from RGD-TSL was not significantly different from Dox delivered from TSL (p-value 0.1).

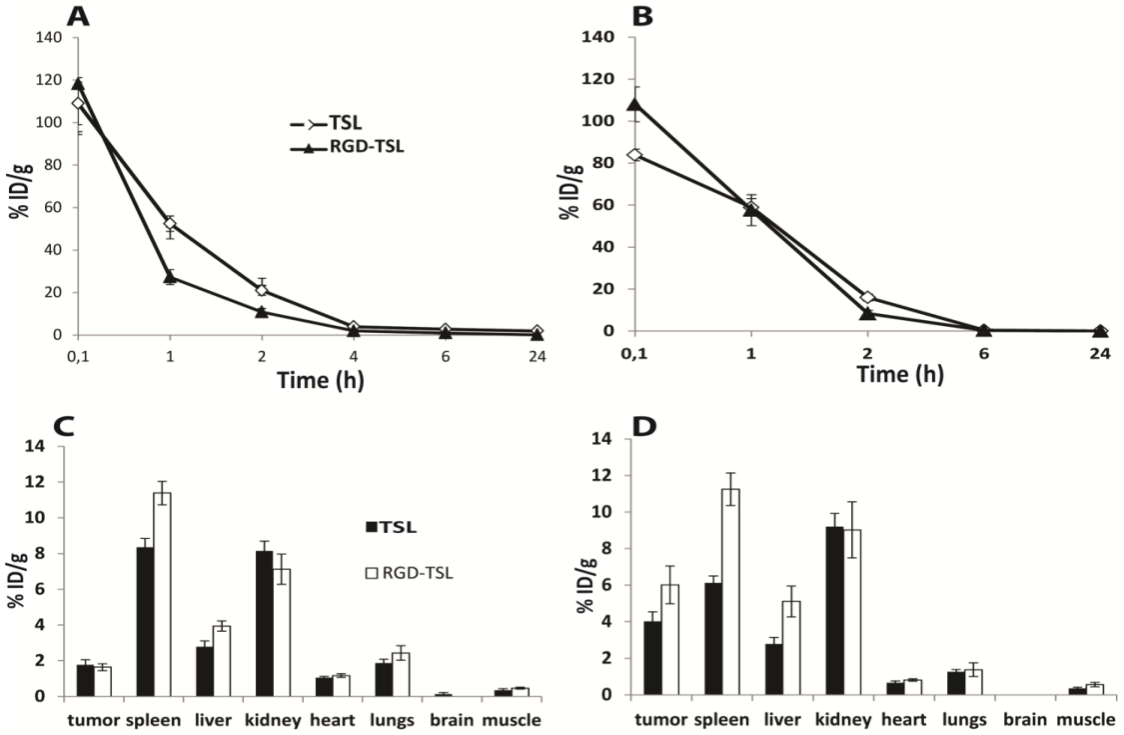

Fig 8. Pharmacokinetics (A and B) and biodistribution (C and D) of Dox-TSL and Dox-RGD-TSL in B16BL6 tumor bearing mice upon NT or initial HT conditions. A,C. At NT condition, mice were injected with 3 mg/kg Dox and blood sampling was performed at the indicated time points and organs collected 24h after liposomes injection. At HT condition (B and D), tumors in mice were preheated for 1h at 41 °C and cooled down for 15min, in order to allow for liposome extravasation. Then,

liposomes were injected at 3 mg/kg Dox and blood samples were collected up to 24h, after which the organs were removed. The Dox concentration in the blood and organs was analyzed by HPLC.

Discussion

Nanoparticles, such as liposomes have been successfully designed and used in treatment of various types of cancer [30]. Although modifications to these nanocarriers contributed to decreased drug-related side effects, their high stability [31-33] and limited tumor localization [34] prevent the desired increase in therapeutic outcome [4, 7]. Forcing encapsulated drug to leave the liposome, as is achieved with TSL exposed to hyperthermia, leads to an enhanced drug release locally in the tumor [35-37]. Also, application of an external trigger such as HT, can promote liposome extravasation from tumor vasculature and increase their accumulation locally in the tumor area [18-20, 38]. When combined, this interstitial release approach relies primarily on HT-augmented liposome extravasation followed by heat-triggered drug release [39]. More directly, improved drug accumulation is observed with the intravascular release approach, for instance with lysolipid-thermosensitive liposomes [40]. Next to the use of an external trigger for controlling drug release, decorating liposomes with targeting ligands specific for the tumor cells or vasculature can also increase the liposomal drug efficacy. We have recently developed cationic thermosensitive liposomes, a nanoparticle combining targeting and triggered release properties in one carrier [41,42] and redesigned it in ordered to achieve a pharmaceutically stable formulation. In the present study, we report on the development of TSL decorated with another targeting ligand, cRGD (RGDf[N-Met]C, which is specific for integrins overexpressed on both tumor vasculature and tumor cells.

Engrafting of TSL with cRGD did not cause significant changes in the pharmaceutical properties of the formulation. Dox-TSL and Dox-RGD-TSL were similar in size, pdI, Dox encapsulation and Dox release kinetics. This is in accordance with Al-Ahmady et al. using monoclonal antibody-targeted thermosensitive liposomes showing that traditional thermosensitive liposomes maintain their physicochemical and thermal properties when conjugated to the monoclonal antibody [43]. Both formulations showed to be stable at temperatures up to 38 °C, slightly released Dox at 39 °C and at 42 °C released >90% of the encapsulated Dox in 1h,characteristics favorable for use in clinical settings. In accordance with Kim et al. [44] and Al-Ahmady et al. [43] there was a preferential uptake of RGD-TSL by both melanoma and endothelial cells, which was confirmed by FACS analysis and confocal microscopy (Fig 3A and B). The specificity of RGD-TSL for the tested cell lines contributed to an increased Dox delivery to all the cell lines at both NT and HT conditions (Fig 3C and D). After 1h of incubation at NT with either TSL or RGD-TSL, there was an increased Dox uptake by all the cell lines from RGD-TSL. HT additionally triggered the drug release and uptake from TSL and RGD-TSL, which was higher for RGD-TSL than TSL. In accordance with Al-Ahmady

et al. [43], the longer incubation period of 3h at 37 °C led to an increased Dox uptake in the cell lines and was higher again for RGD-TSL. However, the subsequent HT trigger did not change the drug uptake in the melanoma cell lines, which is most likely due to the fact that in the 3h period the cells are able to degrade the liposomes themselves preventing an additional release upon HT. Only an increase in drug uptake upon HT after 3h of incubation was observed with HUVECs, which might be due to the fact that these cells take longer time to entrap liposomes in acidic compartments in the cytosol (Fig 4A). In contrast, B16Bl6 and B16F10 were able to process endocytosed RGD-TSL in a faster manner.

Using confocal microscopy, we demonstrated that HT was able to trigger drug release and uptake in vitro in melanoma and endothelial cells. After 3h of incubation of the cells at 37 °C with Dox-RGD-TSL, there was a premature drug release and uptake most likely due to cellular processing of the drug at this time point. However, HT additionally increased Dox delivery in all the cell lines and was mostly nuclear for melanoma cells and both nuclear and cytoplasmic for HUVEC (Fig 4B and C), which is in accordance with Kim et al. [44]. There was no difference in cytotoxicity of Dox-TSL and Dox-RGD-TSL to any of the cell lines at NT conditions.

A HT trigger could not further increase the cytotoxicity of Dox-RGD-TSL in B16F10 and HUVEC but only in B16Bl6. This is likely due to the nature of the assay in which cytotoxicity is measured 72 h after nanoparticle incubation and heat treatment. During the remaining 72 h also non-heat triggered RGD-TSL will release their Dox contents intracellular due to nanoparticle processing, causing cellular cytotoxicity.

The 5h targeting phase did not show any premature Dox release from neither RGD-TSL or TSL (data not shown), which is in accordance with other targeted TSL [42]. During this phase, RGD-TSL bound to tumor endothelial cells already 20min after liposome injection and were observed bound up to 24h. In contrast, TSL did not bind to tumor vasculature and were mostly extravasated 24h after injection (Fig 6A), which confirmed the targeting properties of RGD-TSL. Liposomes were cleared from circulation gradually in 5h, shown by intravital microscopy and image quantification. The application of HT could increase TSL extravasation starting at 30min of HT and increasing up to 1h, which is in accordance with Li et al. [20] and Dicheva et al. [42] (Fig 6B and C).

HT at 42 °C could trigger a massive Dox release from both RGD-TSL and TSL. Release started already 5min after heat trigger and increased up to 1h (Fig 7A). Image quantification from numerous positions in the tumor in several mice demonstrated that due to the RGD-TSL specificity for the tumor, these liposomes delivered 1.7 fold higher amount of doxorubicin than TSL (Fig 7C). However, this difference was not statistically significant. Additionally, hyperthermia triggered release of doxorubicin and subsequent uptake by both tumor and angiogenic endothelial cells, which proves the dual targeting approach (Fig 7D).

The pharmacokinetic behavior of Dox-TSL and Dox-RGD-TSL was investigated with or without initial HT. Initial HT was used to increase liposome extravasation. Dox from RGD-TSL was cleared faster from circulation than Dox from

TSL under NT. This observation might show that targeting influenced Dox clearance. However, upon HT conditions, Dox clearance from circulation from the two formulations was similar.

Biodistribution studies showed that the highest uptake of Dox-TSL and Dox-RGD-TSL was in the spleen and the kidneys followed by the liver (Fig 8C and D). The high spleen and liver uptake are due to the fact that these organs are part of the mononuclear phagocyte system (MPS), which is responsible for filtering out of liposomes from the blood circulation [45]. There was no explanation why kidneys had an increased Dox uptake at both NT and HT conditions. As expected, there was a little Dox uptake from the two formulations under NT and HT in heart and lungs and no uptake in brain and the leg muscle close to the heated tumor. The absence of Dox in the leg muscle shows that the heating was restricted only to the tumor. There was not a difference in tumor uptake of Dox under NT by any of the formulations showing that targeting do not contribute to increased drug uptake at this conditions. However, when initial HT for 1h at 41 °C was applied, there was an increased Dox uptake in the tumor from both formulations, which is likely due to increased extravasation of liposomes upon HT and therefore their higher accumulation at the tumor site.

Future experiments will focus on improving the HT treatment protocols and will address the efficacy of our dual targeted and triggered drug delivery approach using cRGD thermosensitive liposomes.

Conclusion

RGD-TSL encapsulating Dox were successfully developed with both targeting and triggered properties. They demonstrated specificity to tumor and endothelial cells as compared to non-targeted TSL. RGD-TSL were taken up by acidic compartments in cytosol and intracellularly released Dox upon HT. In vivo, RGD-TSL bound to angiogenic endothelial cells and massively released Dox when HT was applied. Biodistribution studies showed that initial HT treatment increases Dox delivery to the tumor from both formulations. Further studies will address the efficacy of our dual targeted and triggered Dox delivery approach using RGD-TSL.

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Chapter 5

Pharmacokinetics, tissue distribution and therapeutic effect of cationic thermosensitive liposomal doxorubicin upon mild hyperthermia

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Abstract

Purpose: To evaluate pharmacokinetic profile, biodistribution and therapeutic effect of cationic thermosensitive liposomes (CTSL) encapsulating doxorubicin (Dox) upon mild hyperthermia (HT).
Methods: Non-targeted thermosensitive liposomes (TSL) and CTSL were developed, loaded with Dox and characterized. Blood kinetics and biodistribution of Dox-TSL and Dox-CTSL were followed in B16BL6 tumor bearing mice upon normothermia (NT) or initial hyperthermia conditions. Efficacy study in B16BL6 tumor bearing mice was followed with Dox-TSL or Dox-CTSL upon NT or HT. Efficacy study in LLC tumor bearing mice was performed upon two HT conditions. Intravital microscopy was performed on B16BL6 tumors implanted in dorsal-skin fold window-bearing mice.
Results: Targeting did not cause faster blood clearance of CTSL compared to TSL. Highest uptake of liposomes was observed in spleen, kidneys and liver. Applying HT prior to CTSL administration increased drug delivery to the tumor and CTSL delivered ~1.7 fold higher Dox concentration compared to TSL. Efficacy in B16BL6 murine melanoma showed that HT had a significant effect on CTSL in tumor suppression and prolonged survival. Efficacy in LLC Lewis lung carcinoma tumor model demonstrates that two HT treatments hold promises for a successful treatment option.
Conclusion: CTSL have potency to increase drug efficacy in tumors due to their targeted and drug release functions.

Keywords: therapeutic efficacy, doxorubicin, cationic thermosensitive liposomes, hyperthermia

Introduction.

Liposomes as one of the best studied nanocarriers for treatment of cancer improve pharmacokinetics and biodistribution of the encapsulated chemotherapeutic drugs after systemic administration [1]. Despite their prolonged blood circulation, in the clinic, pegylated limosomal doxorubicin has only limited therapeutic efficacy [2, 3] due to its low tumor retention and low drug bioavailability [4-6]. An idea to improve this includes targeting of liposomes with specific ligands for increased tumor retention together with an external trigger, i.e heat, which can increase drug delivery locally in the tumor area while preventing the healthy tissues from side effects. The aim of this study was to use cationic thermosensitive liposomes (CTSL) [7] loaded with doxorubicin (Dox), which combine both targeted and triggered characteristics of liposomes in one carrier in order to deal with the drawbacks of the liposomal chemotherapy. The designed nanoparticles made use of shielded cationic lipids for specific recognition of tumor vasculature and tumor cells in combination with thermosensitive lipid bilayers for heat-triggered drug release.

In the clinic, mild hyperthermia (HT) is known to increase the effect of chemo- and radiotherapy leading to enhanced therapeutic efficacy in cancer patients [8,9]. Mild hyperthermia can inhibit DNA repair, augment tissue oxygenation and sensitize cancer cells to cytotoxic drugs [10,11]. Additionally, HT is able to increase blood flow and interstitial fluid flow helping an enhanced passive perfusion of small molecules. More importantly, HT can increase nanoparticle extravasation by increasing the gaps between the vascular endothelial cells [12-14]. In addition, HT can trigger drug release locally in the tumor [13, 15-18]. Previous studies have shown increased therapeutic effect from thermosensitive liposomes (TSL) triggered with mild HT. The effect was mostly due to extravasation and increased drug release locally in the tumor [18-22].

Another approach for improved drug bioavailability comes from active targeting of liposomes to the tumor. Decorating liposomes with ligands specific for tumor vasculature or tumor cells may result in their higher retention in tumors and subsequently increased drug delivery.

Cationic liposomes are known to specifically bind angiogenic endothelial and tumor cells due to the increased expression of negatively charged molecules on these cell membranes [23]. The slower and irregular blood flow in tumors also promotes binding between passing cationic liposomes and tumor vasculature [24]. The specific binding of CTSL to either endothelial or tumor cells may lead to receptor-mediated endocytosis of the carrier therefore bringing the drug closer to the nucleus. CTSL are also composed of thermosensitive lipids with a large capacity to encapsulate drugs and release them upon heat. When HT is applied, CTSL lipid membrane undergoes gel-to-liquid crystalline phase transition and becomes more permeable towards water and solutes [25]. In this way, the encapsulated hydrophilic drugs can be release intracellularly.

Several TSL formulations have been studied [36] and Al-Jamal et al. [37] reported on a detailed study on pharmacokinetics and biodistribution of different TSL in presence or absence of HT. An optimum HT protocol requires knowledge on pharmacokinetics, biodistribution and tumor accumulation of the liposomal nanocarrier as HT may become a treatment option for many types of cancer. However, detailed understanding of the pharmacological behaviour of targeted thermosensitive liposomes is not available yet.

In this study, the pharmacokinetics, biodistribution and therapeutic efficacy of doxorubicin encapsulated in cationic thermosensitive liposomes (CTSL) were investigated. For the efficacy studies, we tested the tumor growth of 2 different types of tumors - B16BL6 murine melanoma and LLC Lewis lung carcinoma over time. The tumor growth of 2 groups – TSL and CTSL was studied with or without HT in B16BL6 or with two HT treatments in LLC . PBS was used as a control.

Materials & Methods.

Chemicals. The phospholipids 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-PEG2000 (DSPE-PEG2000) were purchased from Lipoid

(Ludwigshafen, Germany). The cationic lipid 1,2-dipalmitoyl-3-trimethylammonium-propane (DPTAP Chloride salt) was from Avanti Polar Lipid Inc. Doxorubicin-HCl was purchased from Pharmachemie (Haarlem, The Netherlands). Sodium 3'-[(1-phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) was purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Dioctadecyl tetramethylindotricarbocyanine perchlorate (DiD-C_{18}(3)) was purchased from Invitrogen.

Preparation of TSL. CTSL were composed of DPPC:DSPC:DPTAP:DSPE-PEG_{2000} in a molar ratio 62.5:25:7.5:5. TSL consisted of DPPC:DSPC:DSPE-PEG_{2000} in a molar ratio 70:25:5 All the liposomes were prepared by lipid film hydration and extrusion method. The lipids were dissolved in chloroform and methanol (9:1 vol/vol). Liposomes used for intravital microscopy contained 0.3% of DiD. The solvent was subsequently evaporated under vacuum in rotary evaporator until homogeneous lipid film was formed. The lipid film was hydrated in 250 mM $(NH_4)_2SO_4$ solution at 60 °C for 30 min. The newly formed multilammelar vesicles were extruded subsequently 5 times through 100 nm, 5 times through 80 nm and 5 times through 50 nm polycarbonate filter (thermo barrel extruder at 60 °C) and resulted in small sized TSL. Extraliposomal $(NH_4)_2SO_4$ was removed from liposomal $(NH_4)_2SO_4$ by gel permeation chromatography using a PD-10 Sephadex column (GE Healthcare, Buckinghamshire, UK), eluted with HEPES buffer, pH 7.4 (10 mM HEPES, 135 mM NaCl). Size, polydispersity index (PDI) and zeta potential (ζ) were measured by dynamic light scattering using Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). For size and PDI measurements, TSL were diluted in HEPES, pH 7.4, while the zeta potential was obtained in HEPES, pH 7 without NaCl. Lipid concentration was determined by phosphate assay. [35] After the phosphate concentration was determined, doxorubicin was loaded into the liposomes (5mM lipid) in 0.05:1 drug:lipid ratio (mol:mol) at 38 °C for 1h. The liposomes were concentrated by ultracentrifugation for 2h, 4°C. The pellet was resuspended in HEPES buffer, pH 7.4 and left overnight on slow rotation at 4 °C. Then the liposomes were passed through PD 10 column eluted with HEPES buffer, pH 7.4 to remove residual nonentrapped doxorubicin. Doxorubicin concentration was measured by spectrophotometer at Ex 480nm.

Cell lines and culture. Tumor cell lines B16 (murine melanoma) and LLC (Lewis lung carcinoma) were cultured in a Dulbecco's Modified Eagles' medium (Lonza, Belgium) containing 10% FCS. Cells were subcultured once a week using Trypsin (Sigma, Aldrich) and maintained at 37 °C, 5% CO_2 in a humidified incubator. All experiments were performed at a confluence of 80-90%.

Animal models. The eNOStag-GFP mice line in which the endothelial cells are visible due to constitutive expression of a GFP eNOS-tag fusion protein was used for intravital imaging. Mice weighing about 25 grams were used and fed a standard laboratory diet ad libitum (Hope Farms Woerden, The Netherlands). All animal experiments were done in

accordance with the Dutch law and protocols were approved by the committee on animal experimentation of the Erasmus MC, Rotterdam, the Netherlands.

Preparation of the dorsal skin-fold chamber with B16BL6 tumor is an adaptation from previously described procedures [13, 18, 19]. The mice were housed in an incubation room with an ambient temperature of 30°C and a humidity of 70%. Experiments started 8 to 12 days after tumor implantation, at which a functional vasculature is established in the tumor. For in vivo efficacy study, ~ a 3 mm^3 tumor piece of either B16BL6 or LLC tumors was implanted in the hind limb of C57BL6 mice. Mice were used for experiments when tumors reached ~ 5mm in diameter.

Intravital microscopy for Dox and liposome retention in B16BL6 tumors. DiD-labelled TSL or CTSL containing Dox were injected i.v. (5mg/kg Dox) and let to circulate in the blood stream for 5h in order to allow for liposome targeting to tumor vasculature. After the targeting phase, HT at 42 °C for 1h was applied to trigger drug release from the liposomes. Mice were observed by confocal microscopy (Zeiss LSM 510 META) up to 120h after injection of Dox-TSL or Dox-CTSL in order to visualize Dox and liposome clearance from the tumor. Images of 1024 x 1024 pixels were analyzed using Zeiss LSM image software (Zeiss, Germany), 10x objective lens. Doxorubicin fluorescence was detected by a 543 nm Helium –Neon laser and DiD fluorescence by 613 nm nm Helium –Neon laser.

Pharmacokinetic and biodistribution of Dox-TSL and Dox-CTSL. Pharmacokinetics and biodistribution of Dox-TSL and Dox-CTSL were followed in B16BL6 tumor bearing mice upon NT or initial HT conditions. At NT condition, mice were injected with 3mg/kg Dox and blood sampling was performed at 0.1;1;2;4;6 and 24h and organs were collected 24h after liposomes injection. At HT condition, tumors were first preheated for 1h at 41 °C and then cooled down for 15 min, in order to facilitate liposome extravasation. Then, liposomes were injected at 3mg/kg Dox and blood samples were collected up to 24h (0.1;1;2;6;24h), after which the organs were removed. The Dox concentration in the blood and organs was analyzed by HPLC and calculated as % injected dose/g tissue (%ID/g). 6 mice were used per each group.

Therapeutic efficacy of Dox-TSL and Dox-CTSL in B16BL6 and LLC tumors. C57Bl6 mice were implanted s.c. with B16BL6 murine melanoma or murine LLC Lewis lung carcinoma in their hind limbs. When tumor size reached 5 mm in diameter, mice were anesthetized and the tumor bearing hind legs except the tumor were covered with vaseline to protect them from direct heat. The tumor was in direct contact with the water bath. The hind legs were fixed on a rack to ensure a steady position in a water bath during the HT treatment. Thermocouples were attached to the tumor surface at multiple spots to monitor tumor temperature over time. The water bath temperature was set to 43 °C to reach tumor temperature at 42 °C. In B16BL6 bearing mice, mice were injected with PBS, Dox-TSL and Dox-CTSL (3mg/kg Dox) and 5h later HT at 42 °C for 1h was applied to trigger drug release. Mice with PBS, Dox-TSL and Dox-CTSL

under NT were used as control groups. In LLC bearing mice, there was an initial HT treatment for 1h at 41 °C followed by a cool down for 15 min. Then, liposomes were injected and allowed to circulate for 5h. A second HT for 1h at 42 °C was then applied to trigger drug release. After the treatment, the mice were returned back to the cages. The tumor size and the body weight were measured on the day of the experiment and every other day after the treatment. Mice were sacrificed if the tumor weight exceeded 10% of the body mass, the mice lost 10% body weight, when the tumor reached a tumor size of 1350 mm^3 or at the end of the experiment.

Histology. Mice implanted s.c. with murine B16BL6 melanoma were injected with 3mg/kg Dox-TSL or Dox-CTSL and liposomes were allowed to circulate for 5h. Then, HT for 1h at 42 °C was applied to trigger drug release. Organs and tumors were taken out 24h after liposome injection. PBS without HT was used as a control.

Statistics. *In vivo* biodistribution study was analyzed by Mann-Whitney test and results with p-value ≤ 0.05 were considered statistically significant.

Results.

Pharmacokinetics and biodistribution of Dox-TSL and Dox-CTSL

In order to understand Dox clearance from circulation and its distribution in healthy organs and tumors, pharmacokinetic and biodistribution profiles of Dox in TSL or CTSL were followed (Fig 1A and B) under NT or HT conditions. At both NT and HT conditions, the trend of Dox-TSL and Dox-CTSL clearance from circulation was similar. At NT condition (Fig 1A), Dox from TSL and CTSL seemed to clear from circulation fast in the first 1h (52% and 47% remaining Dox respectively). After 2h of liposome circulation, there were ~ 20% remaining Dox from both formulations. At later time points (4, 6, 24h) there was barely any Dox present in circulation from TSL whereas there were 11% Dox left from CTSL after 4h of circulation. Considering the biodistribution of Dox (Fig 1C and D), at both NT and HT conditions, there was a significant uptake of Dox from the two formulations in spleen as it was significantly higher for Dox from CTSL than TSL under HT conditions (19.7 v/s 6 % ID/g). Similar high Dox accumulation in the kidneys was observed from the two formulations, which was slightly increased upon HT conditions for TSL but significantly increased for CTSL (7.6 to 12.4 %ID/g). Dox accumulated in the liver was slightly higher for CTSL than for TSL under NT (4.7 %ID/g v/s 2.7 %ID/g respectively). However, under HT conditions there was an increase in delivered Dox from CTSL to the liver than TSL (6.7 % ID/g v/s 3.7 %ID/g respectively). The higher Dox uptake in spleen and liver from CTSL is due to most probably opsonization of CTSL by proteins in these organs. There was a minimal uptake of Dox from TSL and CTSL in the heart, lungs and muscle upon NT and HT. No Dox was detected in the brain from neither of the formulations. At NT, the

tumor uptake of Dox was similar for the two formulations. However, the application of initial HT for 1h at 41 °C was able to cause ~ 2.3 fold increased Dox amount to the tumor from TSL (1.7 v/s 4 % ID/g) and 3.4 fold increased Dox to the tumor from CTSL (2 v/s 6.8 %ID/g). The initial HT treatment was able to significantly increase (1.7 fold) Dox delivery to tumor from CTSL compared to TSL.

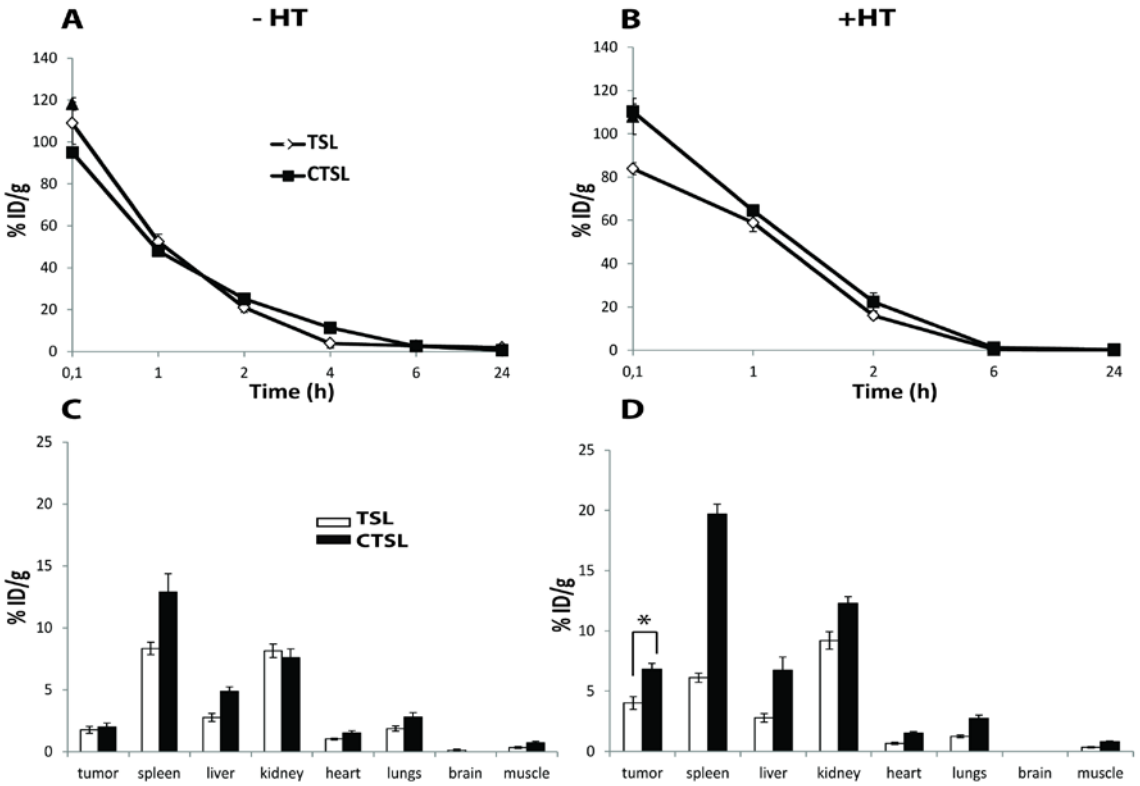

Fig 1. Pharmacokinetics (A and B) and biodistribution (C and D) of Dox-TSL and Dox-CTSL in B16BL6 tumor bearing mice upon NT or initial HT conditions. At NT condition (A and C), mice were injected with 3 mg/kg Dox and blood sampling was performed at the indicated time points and organs collected 24h after liposomes injection. At HT condition (B and D), tumors in mice were preheated for 1h at 41 °C and cooled down for 15min, in order to allow for liposome extravasation. Then, liposomes were injected at 3 mg/kg Dox and blood samples were collected up to 24h, after which the organs were removed. The Dox concentration in the blood and organs was analyzed by HPLC. *Mann-Whitney test, p-value ≤ 0.05.

Tumor growth control and survival of mice with B16BL6 tumors

The efficacy of either Dox-TSL or Dox-CTSL was followed in B16BL6 tumor bearing mice upon either NT or HT conditions (Fig 2A and B). HT itself showed a tremendous effect on tumor growth (A and B). HT effect on the tumor growth was

comparable to TSL without HT. Interestingly, HT added to CTSL decreased significantly tumor growth compared to their effect on the tumor growth without HT. HT applied to TSL did not add to inhibiting tumor progression. However, the combination of liposomes and HT showed the highest therapeutic effect. In the CTSL plus HT group, 4 out of 8 mice survived 12 days post-treatment whereas in the TSL plus HT group, 5 out of 6 mice survived 10 days post-treatment (C). In comparison, the group with CTSL without HT survived only 8 days post-treatment (4 out of 8 mice). Therefore, HT applied to mice treated with CTSL increased their survival by 4 days. HT added to TSL did not increase mice survival. HT added to PBS increased survival only with 1 day.

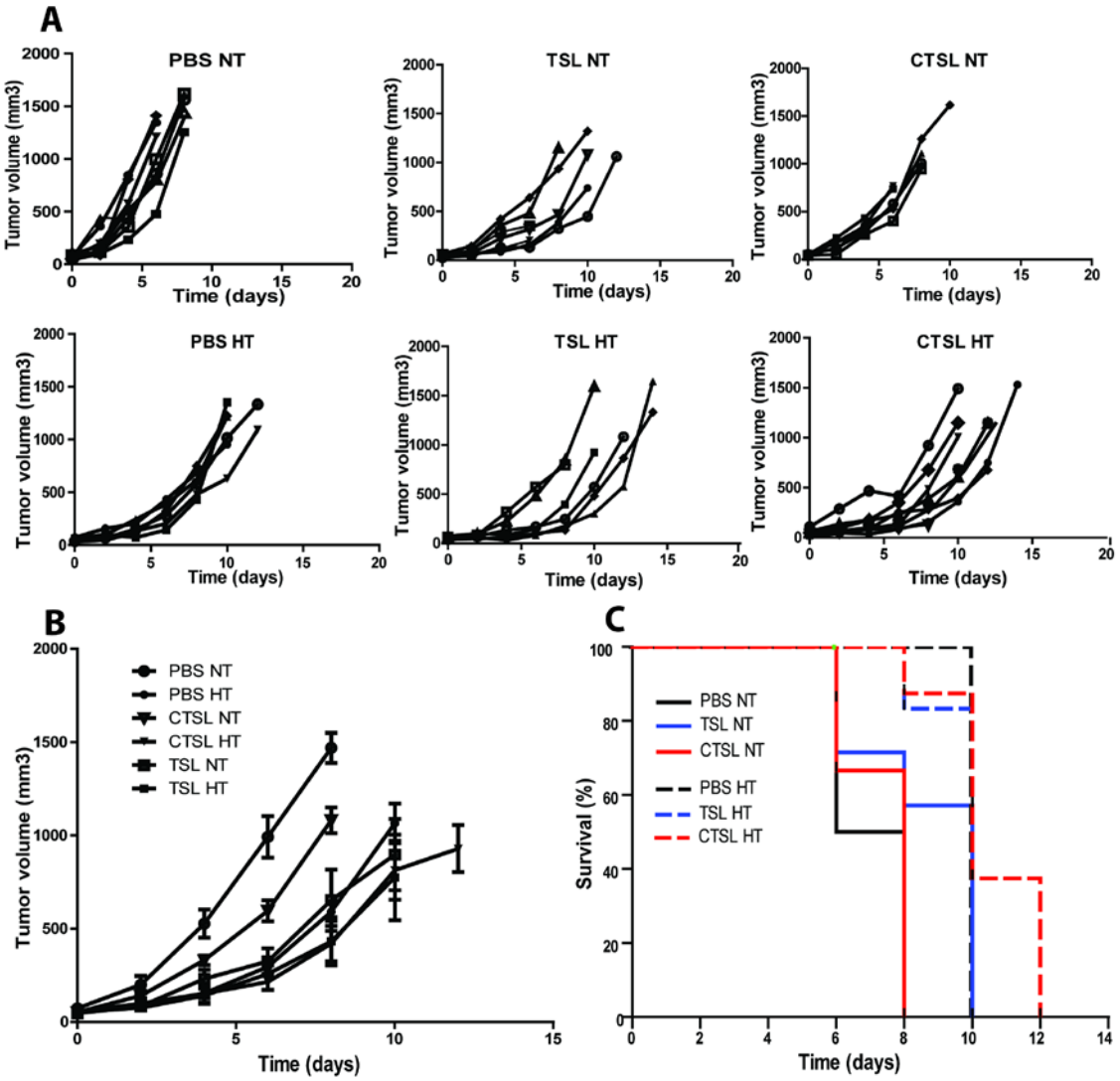

Fig 2. Mice implanted with B16BL6 tumors were injected with 3mg/kg Dox-TSL or Dox-CTSL. In the HT group, liposomes were allowed to circulate for 5h, after which

HT at 42 °C for 1h was applied to trigger drug release. A. Individual tumor growth curves from all mice in all treatment groups. B. Efficacy of all treatments. C. Survival of mice upon different treatments.

Tumor growth control and survival of mice with LLC tumors

The efficacy of Dox-TSL and Dox-CTSL was followed in mice implanted with LLC tumor model based on two different treatment schedules: - preheating of the tumor for 1h at 41 °C followed by cooling it down for 15 min, injection of liposomes, allowed to circulate for 5h and subsequently application of HT for 1h at 42 °C or: - the same treatment skipping the preheating phase (Fig 3). The preheating phase was used to induce extravasation of liposomes. Treatment with initial HT additionally to one HT treatment decreased significantly tumor growth and prolonged survival only in the case of Dox-CTSL (Fig 3A and B). Survival was increased with 8 days (from 10 to 18 days). The effect on tumor growth of Dox-TSL and Dox-CTSL with preheat were similar. Dox-CTSL with preheat increased the survival of mice with 2 days (from 16 to 18 days) compared to Dox- TSL with preheat. Preheating phase did not add to one HT treatment in inhibiting tumor growth or increasing survival when Dox-TSL or PBS alone were used.

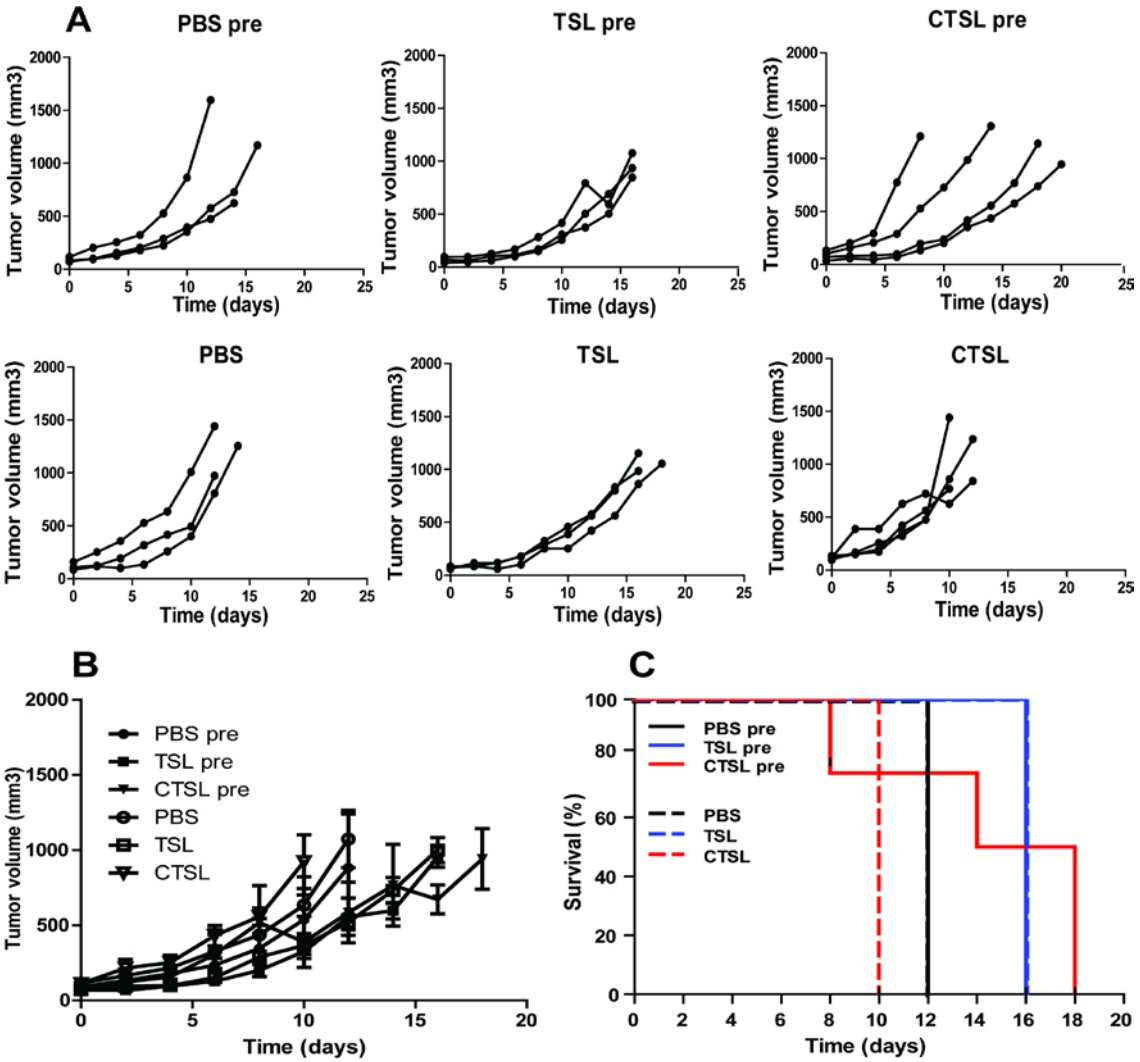

Fig 3. Mice implanted with LLC tumors were either preheated for 1h at 41°C followed by 15 min of cooling down to body temperature or were not preheated. Then, they were injected with 3mg/kg Dox-TSL or Dox-CTSL, after which the liposomes were allowed to circulate for 5h at NT. After that, HT at 42 °C for 1h was applied to trigger drug release. A. Individual tumor growth curves from all mice in all treatment groups. B. Efficacy of all treatments. C. Survival of mice upon different treatments.

Control on treatment toxicity in mice with B16BL6 tumors

Dox-TSL and Dox-CTSL effect on mice regarding their toxicity was tested by measuring body weight every other day after treatment. In B16BL6 tumor model, PBS and PBS plus HT treatment did not show any toxicity on mice. All the other treatments with or without HT demonstrated toxicity only in the first two days after treatment shown by drop in body weight. However, after 2 days, body weight of all mice from all

treatment groups was recovered and remained stable until death (Fig 4A). Similarly, in LLC tumor model, there was an initial body weight loss in all the tratment groups, which was recovered in 2 to 4 days after treatment and remained stable until death of mice (Fig 4B).

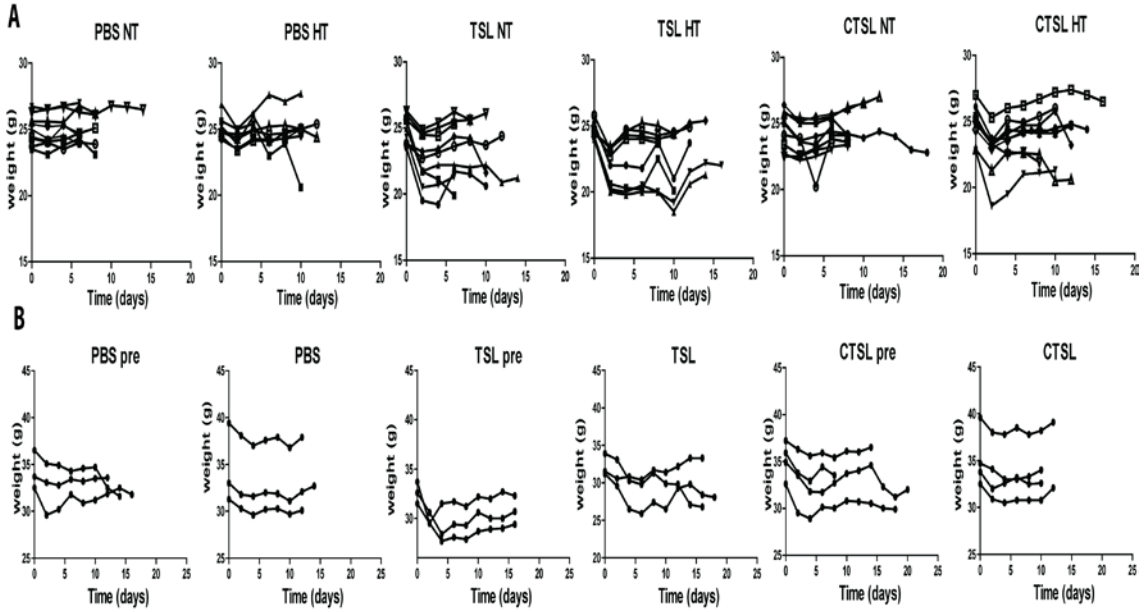

Fig 4. Body weight of the treated mice was followed every other days after treatment until death. A. Body weight of mice with B16BL6 tumors. B. Body weight of mice with LLC tumors.

Intravital microscopy on liposome and Dox distribution in tumors

In order to know which formulation of liposomes will be more effective in killing the tumor, we followed the retention of liposomes and Dox in tumor. Images of B16BL6 window chamber tumor bearing mice were taken up to 5 days after DiD labelled Dox-CTSL or Dox- Dox-TSL injection (Fig 5). Images show that the two formulations extravasated from circulation after 24h and can be found around the tumor vasculature or associated with it in the case of CTSL. Liposome and Dox clearance from the tumor progressed over time as can be concluded from decreased DiD and Dox fluorescent signal in the tumor. Remarkably, there was still an abundant amount of liposomes left in the tumor tissue even 120h after injection of the two formulations. The Dox clearance from the tissue was faster for TSL (B) than for CTSL (A) which is suggestive for higher retention of CTSL in tumors.

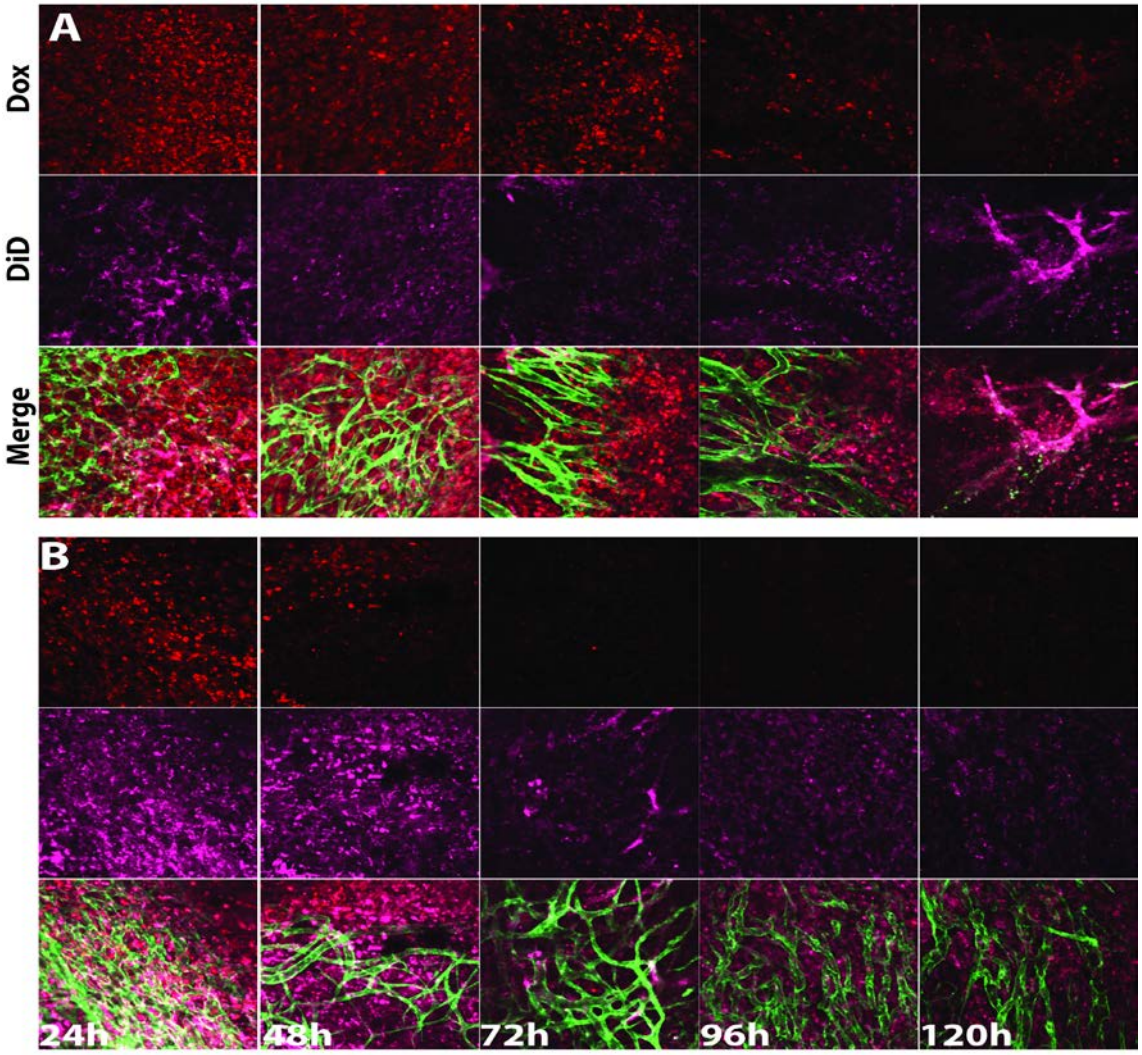

Fig 5. Doxorubicin and liposome retention in B16BL6 tumors implanted in window chamber bearing mice. Mice were injected with 5mg/kg Dox-CTSL (A) or Dox-TSL (B) labelled with DiD. Liposomes were allowed to circulate for 5h, after which HT was applied to the tumor for 1h. Mice were observed up to 120h in order to follow up the Dox and liposomes clearance from the tumor.

IHC of tumor and normal tissues

Dox-CTSL plus mild HT for 1h at 42 °C caused interstitial haemorrhage in s.c. murine B16BL6 melanoma. Oedema was also seen in this treatment group. No obvious pathology was obserbed in tumors from mice from the other groups. None of the treatments showed any toxicity to the normal organs as concluded from the morphology of the spleen, kidneys and liver compared to the control PBS treatment.

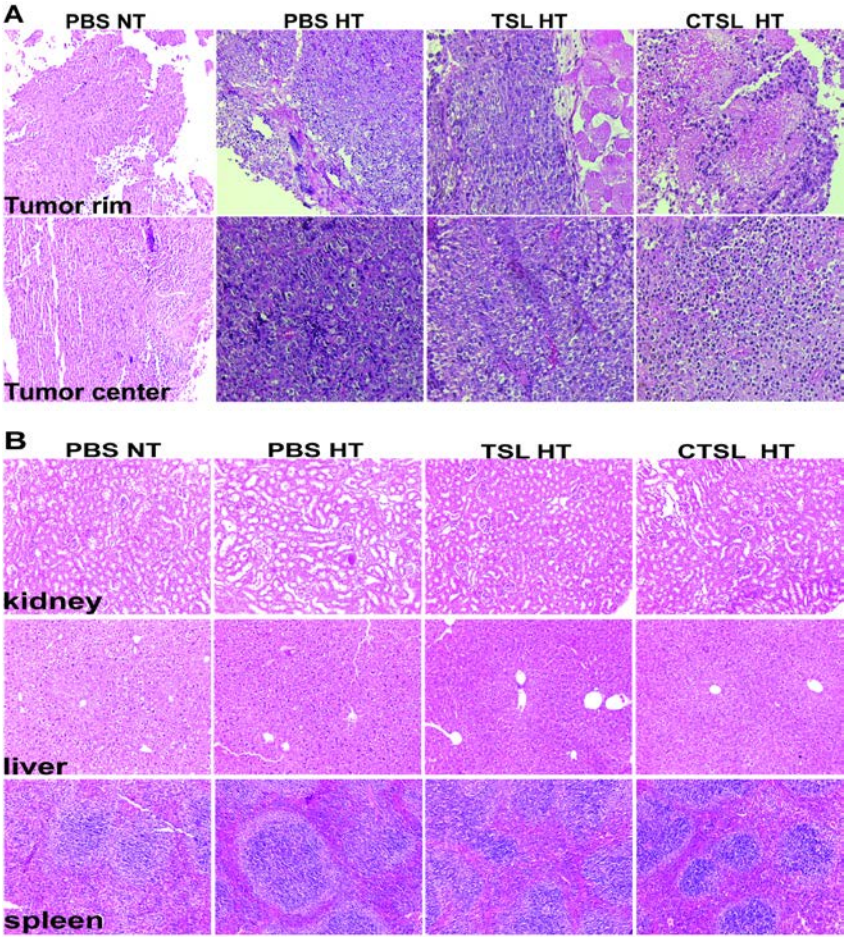

Fig 6. H&E staining of s.c. murine B16BL6 melanoma tissues (A) in tumor rim or tumor center and in normal tissues (kidney, liver and spleen) (B) treated with PBS, Dox-TSL or Dox-CTSL under HT.

Discussion.

Thermosensitive liposomes loaded with Dox in combination with HT have emerged as a promising treatment approach for cancer patients [39-41]. The aim of this approach is to increase drug levels in the tumor, thus increasing therapeutic efficacy. Several TSL formulations have been developed in the last two decades differing in their serum stability, thermosensitivity and ligand targeting [7, 17, 20, 42-45]. Liposome formulations having stability at 37 °C and fast release kinetics at HT will offer best results in the clinic. For optimal application, stability of liposomes at physiological

temperatures is very important in order to prevent premature drug release. Despite the many efforts made until now [42, 46, 47], the search for the best liposomal formulation is still ongoing. Reasons for this can be the limited tumor accumulation and specificity of liposomal nanoparticles, low drug bioavailability due to its stable entrapment and lack of control of drug release. Many novel approaches have been proposed in the past years to tackle these issues and thereby improve liposomal chemotherapy. We used two key approaches for improvement in this study being cell-specific targeting and temperature-controlled drug release. On one hand, CTSL are positively charged and will recognize negatively charged anionic sites on the membranes of tumor endothelial cells and tumor cells. In this way they can target both tumor vasculature and tumor cells. Once in contact, the cationic lipids from CTSL will bind to the anionic molecules on the tumor or endothelial cells. This binding might evoke a receptor-mediated endocytosis, leading to CTSL internalization. On another hand, CTSL contain thermosensitive lipids in their bilayer and when HT is applied to trigger drug release, areas with increased permeability in the membrane of CTSL will be created, thus allowing the transport of the chemotherapeutic drug through them. This intracellularly released drug from the internalized CTSL will then be transported to the nucleus where it can exert its therapeutic effect.

Although many studies focused on determination of Dox levels in tumors and blood and a comprehensive study by Al-Jamal et al. [37] described the pharmacokinetics and biodistribution of different TSL with or without HT, insight into biodistribution and pharmacokinetics of targeted TSL is still lacking. Therefore, this study focuses on understanding these together with the therapeutic efficacy of our CTSL.

The pharmacokinetic behavior of Dox-TSL and Dox-CTSL was investigated with or without HT. In accordance with Al-Jamal et al. [37], local HT did not affect the blood kinetics of Dox from TSL and CTSL and it was cleared from circulation in a similar manner both under NT and HT conditions (Fig 1A and B). After 1h of liposome circulation under NT, ~50 % of the encapsulated Dox was cleared, after which its concentration gradually decreased. This pharmacokinetic profile of CTSL proves that targeting does not cause faster liposome and subsequently drug clearance from circulation and it is in accordance with Dicheva et al [7] showing by intravital microscopy that the concentration of fluorescently labelled CTSL and TSL in circulation was similar. Fig 5 confirms that targeting does not lead to faster liposomal clearance and shows that targeting contributes to a longer liposomal and drug retention in tumors. Dox from CTSL showed the only presence in circulation 4h after injection, whereas Dox from TSL was completely cleared. At later time points, Dox levels were below detection in any of the formulations for both NT and HT.

Biodistribution studies showed that the highest uptake per gram of tissue of Dox-TSL and Dox-CTSL was in the spleen and the kidneys followed by the liver (Fig 1B). This observation is also in accordance with Al-Jamal et al. [37] showing the highest uptake of their formulations in liver and spleen. The high spleen and liver uptake are due to the fact that these organs are part of the mononuclear phagocyte system (MPS), which is responsible for filtering out foreign particles from the blood circulation [48].

There was no explanation why kidneys had an increased Dox uptake at both NT and HT conditions. Interestingly, HT increased Dox from CTSL in spleen, kidneys and liver. As expected and in accordance with Al-Jamal et al.[37], there was a little Dox uptake from the two formulations under NT and HT in heart and lungs and no uptake in brain and the leg muscle close to the heated tumor. The absence of Dox in the leg muscle shows that the heating was restricted only to the tumor. There was no difference in tumor uptake of Dox under NT from both formulations showing that targeting does not contribute to increased drug uptake at this condition. However, when initial HT for 1h at 41 °C was applied, there was an increased Dox uptake in the tumor from both formulations, which is likely due to increased extravasation of liposomes upon HT and therefore their higher accumulation at the tumor site. Additionally, Dox concentration from CTSL in the tumor was significantly higher compared to TSL, which is most likely due to the targeting nature of CTSL causing a higher accumulation of the carrier in the tumor and subsequently increased drug delivery.

In B16BL6 tumor model, HT itself had a tremendous effect in decreasing tumor growth (Fig 3) but also increased survival as compared to only PBS treatment. HT as an additive treatment to liposomes had a great effect on CTSL in reducing tumor progression compared to TSL. In this case, the survival was increased from 8 to 12 days, whereas in the case of TSL the survival was not increased. There was not a significant difference in tumor growth inhibition between mice treated with Dox-TSL HT and Dox-CTSL HT, which shows that in this tumor model the targeting does not play a role in reducing the tumor volume compared with a non-targeted formulation.

As the efficacy study with B16BL6 did not show the benefit of using targeted thermosensitive liposomes in inhibiting tumor growth, LLC tumor model was included in a pilot study where two HT treatments were used - an initial mild HT at 41°C for 1h to induce permeable tumor vasculature for liposome extravasation and; a second heat to trigger drug release [49]. It was recently reported by Li et al. that a temperature of 41 °C for 1h can cause significant liposome extravasation in multiple murine and human tumor models [14]. As seen in Fig 3, the two HT treatments led to reduced tumor growth by Dox-CTSL compared to one HT treatment. The two HT treatments were most efficacious for Dox-CTSL showing increased survival from 10 to 18 days. Preheating phase had no effect on TSL and PBS when compared to one HT treatment.

Interestingly, histology demonstrated that only CTSL plus HT could cause hemorrhage and edema in the treated mice. This observation is in accordance with Dicheva et al. [7] demonstrating massive vessel destruction at 24h after liposomal injection when CTSL are used in combination with HT. In the treated tumor models, HT showed the highest effect in tumor suppression as an additive to Dox-CTSL compared to Dox-TSL. This might be a result of its higher stability in serum leading to an increased levels of released drug upon HT. Another factor contributing to it might be that HT increases CTSL binding to endothelial cells [44] leading to its higher retention and effectiveness in tumor growth inhibition. However, more comprehensive studies about liposome pharmacokinetics are necessary. Interestingly, two HT treatments might have a better treatment result with targeted liposomes than one HT treatment. While

intravascular release approach is considered to provide better results with non-targeted liposomes, the results presented here indicate a possible application for the so-called two step approach where HT is used to open up tumor vessels and to trigger release from targeted liposomes.

Conclusion.

Targeting of TSL did not lead to increased clearance of CTSL from circulation compared to TSL. Initial HT condition increased Dox uptake in tumors from CTSL compared to TSL. Efficacy study in B16BL6 tumor model demonstrated that HT had a significant effect on CTSL on tumor inhibition and prolonged survival. Efficacy study in LLC tumors showed that two HT treatments hold promises for successful therapeutic efficacy.

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Chapter 6

Targeted thermosensitive liposomes: an attractive novel approach for increased drug delivery to solid tumors

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Based on:
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Abstract. Introduction. Currently available chemotherapy is hampered by a lack in tumor specificity and resulting toxicity. Small and long-circulating liposomes can preferentially deliver chemotherapeutic drugs to tumors upon extravasation from tumor vasculature. Although clinically used liposomal formulations demonstrated significant reduction in toxicity, enhancement of therapeutic activity has not fully met expectations. **Areas covered.** Low drug bioavailability from liposomal formulations and limited tumor accumulation remain major challenges to further improve therapeutic activity of liposomal chemotherapy. The aim of this review is to highlight strategies addressing these challenges. A first strategy uses hyperthermia and thermosensitive liposomes to improve tumor accumulation and trigger liposomal drug bioavailability. Image-guidance can aid online monitoring of heat and drug delivery and further personalize the treatment. A second strategy involves tumor-specific targeting to enhance drug delivery specificity and drug internalization. In addition, we review the potential of combinations of the two in one targeted thermosensitive triggered drug delivery system. **Expert opinion.** Heat-triggered drug delivery using thermosensitive liposomes as well as the use of tumor vasculature or tumor cell-targeted liposomes are both promising strategies to improve liposomal chemotherapy. Preclinical evidence has been encouraging and both strategies are currently undergoing clinical evaluation. A combination of both strategies rendering targeted thermosensitive liposomes (TTSL) may appear as a new and attractive approach promoting tumor drug delivery.

Keywords: targeted thermosensitive liposomes, hyperthermia, triggered release, drug delivery

1. Introduction. Cancer chemotherapy is an important treatment option for primary (non-resectable) or metastatic tumors. Administration of chemotherapeutic drugs usually occurs systemically, but its efficacy is greatly hampered by rapid drug clearance from circulation, lack of tumor specific delivery and activity and distribution of the drug throughout the body. The latter is responsible for toxicity to normal organs and tissues, usually limiting drug dosing. Due to these factors, only suboptimal levels of active drug will eventually reach the tumor limiting anti-tumor activity. In order to overcome these disadvantages of currently available chemotherapy, the encapsulation of drugs in a biocompatible nanoparticle such as a liposome, may lower systemic exposure and thus toxicity and on the other hand help to improve tumor-specific drug delivery. In this way liposomal chemotherapy will contribute to an increased therapeutic index, i.e. increased efficacy over a lower toxicity.

Liposomes, self-assembling nanovesicles consisting of a lipid bilayer enclosing an aqueous phase, represent one of the best studied nanomedicines used in the treatment of cancer. Currently several liposomal products have obtained approval for clinical use such as: liposomes with doxorubicin – (Doxil/Caelyx, Myocet and Lipo-Dox) for treatment of Kaposi's sarcoma, ovarian cancer, breast cancer and multiple myeloma, liposomes with daunorubicine (DaunoXome) for treatment of Kaposi's sarcoma and

liposomes encapsulating vincristine – (Marqibo) for acute lymphoblastic leukemia [1]. Many more liposomal drugs for cancer treatment have reached various stages of clinical trials, such as Endo-Tag-1 with paclitaxel as an active compound for treatment of pancreatic cancer and triple negative breast cancer; Brakiva loaded with topotecan for relapsed solid tumors; Lipoplatin with cisplatin as an active drug for non-small cell lung cancer [1]. Liposomes have numerous advantages as drug carriers over the free drug. In their aqueous phase water soluble drugs can be entrapped, whereas the lipid bilayer can be used to incorporate lipophilic drugs. A drug that is encapsulated in pegylated liposomes, in which the liposome surface is coated with polyethylene glycol (PEG), has a greatly altered pharmacokinetic profile, including the area under the plasma concentration-time curve, the circulation half-life, the volume of distribution and clearance. These parameters greatly contribute to a limited drug distribution into healthy tissue while increasing it concentration at the tumor site [2]. Anthracyclines are the best studied group of drugs which have been associated with liposomes. In preclinical models, Doxil/Caelyx produced remission and cure against many types of tumors including tumors of the breast, lung, ovaries, prostate, colon, bladder, and pancreas, as well as lymphoma, sarcoma and melanoma[1]. Whereas distribution of liposomal drug is mainly restricted to the vascular system, in tumors this is different. If liposomes are long-circulating and small enough they can pass through tumor capillaries characterized by increased permeability [3, 4]. This process has been addressed as the enhanced permeability and retention effect [4]. Tumor vessels are leakier due to ongoing angiogenesis and related basement membrane abnormalities and decreased numbers of pericytes lining the endothelium. Gap sizes in the discontinuous tumor endothelium can range from 100 to 400 nm depending on the tumor type and even within a tumor [3,5] . This is in contrast to the tight endothelial lining of normal vessels, preventing liposomal drug extravasation at those sites. Permeable tumor vessels allow extravasation of small liposomes into the interstitial space to reach tumor cells. However, this extravasation process is not a general phenomenon and appears relatively heterogeneous within a tumor and also highly variable between different tumor types [6]. For instance, imaging radiolabeled liposomes as done by Harrington et al. clearly demonstrated that tumor accumulation of liposomes in cancer patients strongly depends on tumor type and size [7]. In this study was shown that the levels of liposome uptake in tumors estimated from regions of interest on gamma camera images were approximately 0.5–3.5% of the injected dose at 72 h. The greatest concentration of liposomes (33% ID/kg) was found in head and neck tumors, followed by lung tumors (18.3% ID/kg) and breast tumors (5.3% ID/kg).

Although liposomes can accumulate in tumors to some extent their non-tumor-specific nature and high intrinsic stability hinders further drug bioavailability. Drug release from liposomes is usually gradual and inefficient, causing low drug uptake in tumor cells. Seynhaeve et al. demonstrated slow uptake of liposomal doxorubicin by tumor cells with evidence of nuclear delivery of bioavailable drugs only 2-3 days following systemic administration [8]. The low drug bioavailability from liposomal formulations together with their limited accumulation in tumors in cancer patients are

thought to be the main reasons for the absence of major improvements in therapeutic outcome when applying liposomal doxorubicin [9].

Summing up, liposomal formulations of chemotherapeutic drugs can lower toxicity to normal tissues, however improving their specific accumulation in tumors and promoting drug bioavailability to tumor cells remain important challenges to further improve their therapeutic activity.

Liposomal drug bioavailability can be improved by several means including heat-triggered release from thermosensitive liposomes (TSL) or promoting cellular internalization by receptor-specific targeting.

In the first approach heat is used to trigger drug release from thermosensitive liposomes (TSL) locally in tumors thereby improving drug bioavailability [10,11]. TSL contain thermosensitive lipids in their bilayer undergoing a gel-to-liquid phase transition at the desired temperature (usually between 40 and 45 °C) causing release of entrapped hydrophilic drugs [12]. The drug delivery approach using TSL primarily relies on release of the drug at the tumor site, after which the drug can enter tumor cells in free form.

The second approach aims to improve liposomal drug bioavailability through the use of cell specific targeting ligands attached to the nanoparticles. First of all these targeting ligands can achieve (cell-) specific localization and retention of the liposomal drug in tumors e.g. on tumor cells or tumor vasculature. Second, these ligands can promote active cellular uptake of the drug-containing nanoparticles through binding to targeted internalizing receptors. By these means the drug is transported across the cell membrane, which normally forms a significant barrier for cellular drug uptake. Combining such targeting functions of ligands with the pharmacokinetic and drug delivery advantages of liposomes, targeted liposomes represent promising, tumor specific drug delivery nanoparticles [13-21].

In this review we aim to highlight those two important approaches to improve liposomal chemotherapy that aim at increasing drug bioavailability through triggered drug release or by using tumor-specific targeting to enhance tumor specificity and promote drug internalization. In addition, we review the potential of a combination of those two approaches in one targeted thermosensitive triggered drug delivery system.

2. Hyperthermia

Mild hyperthermia, the application of temperatures a few degrees higher than physiological temperatures (40-44 °C) to tumor tissue, has become an attractive treatment strategy to enhance the efficacy of radio- or chemotherapy in the treatment of cancer patients.

Initially, whole body hyperthermia was applied to the patient for homogeneous tissue temperature distribution. However, in this approach the high core temperature (>40 °C) was not very well tolerated by the patient [22]. An improvement of the hyperthermia treatment involves the application of local hyperthermia by concentrating electromagnetic or ultrasound energy on the tumor coming from water-filled waveguides or ultrasound transducers, respectively [23].

Despite the achievements in hyperthermia treatment planning and local heat application, there are still obstacles concerning spatial accuracy and deep thermal response in solid tumors. Recent studies report on MRI-guided hyperthermia [24-26] and MRI-guided HIFU [27-29] (high intensity focused ultrasound), where MRI (magnetic resonance imaging) provides information on tissue anatomy for better treatment control and temperature mapping. MRI-guided hyperthermia helps to direct the heat to the desired region by online MR thermometry, thereby achieving higher treatment efficiency. Whereas electromagnetic hyperthermia has been used for both mild hyperthermia and tumor ablation, the most common application for HIFU is thermal ablation at temperatures >60 °C. More recently the application of HIFU for mild hyperthermia has also been demonstrated in preclinical studies in combination with TSL [23, 27, 28].

Hyperthermia (HT) plays a role as a potent radiosensitizer by increasing tumor perfusion and oxygenation causing enhanced radiosensitivity [30]. Mild hyperthermia as an adjuvant therapy to radiotherapy or chemotherapy has become a standard clinical treatment option for various tumor types and has shown increased survival in several randomized clinical trials [31-36].

The combination of HT and chemotherapy has also proven beneficial in various clinical trials [37, 38]. Hyperthermia can cause cellular sensitization and thus cytotoxicity towards some chemotherapeutic drugs, especially platinum drugs [39-42] whilst doxorubicin has a temperature threshold beyond which its cytotoxicity can be increased [41]. al-Shabanah et al. stated that the application of HT at 43 °C for 30 min could enhance the cytotoxicity of doxorubicin to Ehrlich ascites carcinoma cells [40]. A study by Urano et al. using spontaneous mouse fibrosarcoma cells compared the cytotoxicity of HT at 43 °C alone for 1h versus 1h of HT in combination with doxorubicin and found that in this period there was no difference in cytotoxicity. However, the prolonged treatment caused increased cytotoxicity in the combination group, which can suggest that doxorubicin cytotoxicity can be increased if tumors are subjected to the hyperthermia treatment for prolonged time or at elevated drug doses [42]. However, most of those studies refer to thermal sensitization in vitro at elevated temperatures which are difficult to achieve in clinical settings. Furthermore, the thermal sensitization of a drug depends on the administration mode and dosage [41]. An additional effect of HT combined with chemotherapy is the recently discovered effect on DNA-repair inhibition [43-45]. Radiation and chemotherapy action is often based on induction of DNA double strand breaks (DSB). Under normal circumstances, effective DNA repair mechanisms are executed to protect against abnormal DNA rearrangements. Two main mechanisms involved in DSB repair are homologous recombination (HR) and non-homologous end joining. HR was demonstrated to be inhibited by hyperthermia through degradation of BRCA2 [43-45]. As a result innately HR-proficient BRCA2-positive tumors became sensitive to PARP-1 inhibitors through hyperthermia. In this approach hyperthermia had no direct effect on DNA damage, but rather on the proteins involved in DNA repair, replication or chromosomal segregations [45].

Hyperthermia in combination with chemotherapy has proven to be an effective treatment option for patients with localized soft-tissue sarcoma as was shown recently in a large phase III clinical trial [38]. This can be explained, not only by the cellular sensitizing effect of HT, but also by its effects on tumor pathophysiology. HT has been shown to increase tissue perfusion, oxygenation, blood flow velocity and microvessel permeability contributing to increased drug levels in tumors at clinically tolerated temperatures [46, 47]. HT is also known to increase tumor vessel permeability to antibodies [48] and ferritin [49] and even more interesting, to nanoparticles [4, 50-52].

HT is able to induce changes in vessel morphology leading to increased endothelial cell gap size where the nanoparticles may extravasate efficiently from the circulation. Pioneering work by Dewhirst's group reported on increased extravasation of liposomes when s.c. implanted human ovarian carcinoma were subjected to HT at 42 °C whereas they remained impermeable for liposomes at 34°C [49-51]. Recently Li et al [52] demonstrated that in contrast to NT, a temperature of 41 °C for 1h was able to increase vessel permeability to liposomes in 4 different tumor types (murine B16 melanoma, BFS-1 sarcoma, LLC Lewis Lung Carcinoma and human BLM melanoma), but not in normal vessels. Moreover, this study demonstrated that HT caused significant liposome penetration in tumor tissue up to at least 27 μm from the tumor vessels. Vascular hyperpermeability remained for several hours after HT of which the duration varied in the different studies between 6 h [51], 8 h [52] and 24 h [53]. The work by Kong et al in human ovarian carcinoma implanted in skin-fold window chamber bearing nude mice reported on 2-4 fold increases in liposomal uptake in heated tumors than in non-heated [51,54]. Moreover, studies in cats with soft-tissue sarcoma showed 2-16 fold increase of liposomal drug delivery due to hyperthermia [55]. Hyperthermia also increased the extravasation of small molecule drugs leading to increased drug uptake [56] and nanoparticulate systems such as polymers [57]. Interestingly in the latter study, heat cycling (repeated hyperthermia treatments followed by cooling) caused an even further increased macromolecular drug carrier accumulation in the FaDU (human squamous cell carcinoma) tumors compared to a single hyperthermia treatment [57]. Additionally, HT applied to elastin-like polypeptides-Dox was able to completely inhibit tumor growth compared to the free drug [58]. Moreover, in a recent study by Bidwell et al. was shown that temperature-responsive polypeptide, which is based on elastin-like polypeptide, was able to inhibit tumor growth in a mouse model of breast cancer [59]. Local induction of hyperpermeability can induce tumor selective liposome extravasation which is an important advantage of combining hyperthermia and liposomal chemotherapy in cancer treatment. An additional advantage comes from the use of HT to trigger release from thermosensitive liposomes in the tumor as will be discussed in the next section. In summary, hyperthermia has shown a prominent effect when applied in combination with radio-or chemotherapy in a multifactorial manner and is now an established treatment modality for patients with various types of cancer enhancing the effectiveness of radio and/or chemotherapy with limited to no side-effects [34-38]. Novel therapies involving nanoparticle-mediated drug delivery provide attractive options for combination with hyperthermia benefitting the drug delivery process.

3. Thermosensitive liposomes

Besides the above discussed benefits on drug accumulation, increased cellular sensitivity and improved nanoparticle delivery, hyperthermia may also be used to trigger drug release from liposomes composed of thermosensitive lipids. The idea of using TSL and HT for drug delivery was proposed in 1978 by Yatvin and Weinstein, who designed liposomes consisting of the thermosensitive lipids DPPC and DSPC undergoing gel-to-liquid phase transition at temperature (Tm) around 44 °C [60]. DPPC undergoes gel-to-liquid transition at 41.3°C, at which the passive permeability of ions is enhanced. To fine tune drug release temperature and/or drug release rate DPPC can be mixed for instance with defined quantities of for example Lyso-PC [11], DSPC [27,60-62], DPPGOG [63] or PEG2000-DSPE [62]. Thus, upon heat areas with increased permeability will occur in the bilayers of these TSL, allowing release of encapsulated drug (**figure 1**).

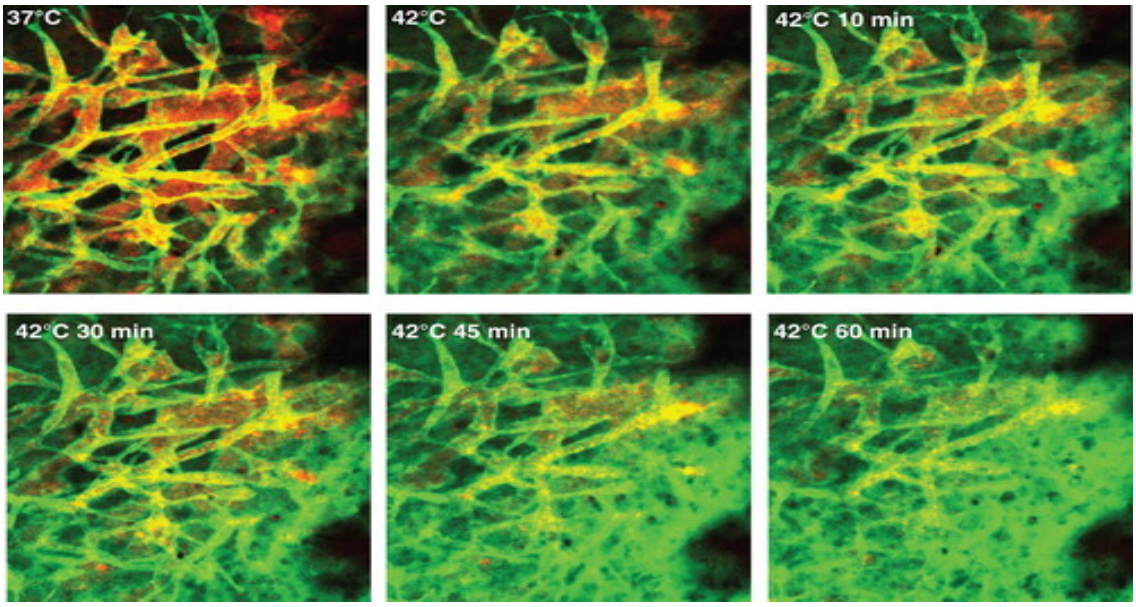

Fig 1. Release of an entrapped hydrophilic marker (CF) from cationic thermosensitive liposomes into the tumor tissue upon hyperthermia. At 37 °C thermosensitive liposomes are stable, not releasing their content. Once heated, they release the encapsulated marker (green) into the surrounding tumor tissue.

The initially described TSL underwent additional modifications in order to be stabilized in blood circulation by adding additional membrane components, such as HSPC and cholesterol [64, 65]. Although stability was increased, these adaptions either compromised the desired transition temperature applied by mild hyperthermia (41-43 °C) (DSPC and HSPC) or led to formulations that were no longer responsive to temperature change (high cholesterol content) [65]. Further improvements of thermosensitive liposomes were achieved by decorating them with polyethyleneglycol

or oligoglycerol moieties for prolonged circulation [62, 63] and incorporating additional lipids e.g. lysolipid [11] or oligoglycerol-PG [63] in their membrane to further increase their membrane permeability at the phase transition temperature.

Low temperature sensitive liposomes, LTSL, is the first heat-triggered release formulation of the anthracycline doxorubicin that reached pharmaceutical development (ThermoDox ®, Celsion Corporation, Columbia, Maryland, USA) and clinical application, and was originally developed by Needham and Dewhirst's groups [11,66]. The bilayer of this formulation is composed of the synthetic phospholipids DPPC (1,2-dipalmitoyl-sn-glycero-phosphatidylcholine), MSPC (monostearoyl-sn-glycero-phosphatidylcholine), and DSPE-MPEG-2000 (1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine-N-[methoxy(polyethyleneglycol)-2000] where the presence of the lysolipid MSPC is thought to cause formation of pores in the liposomal bilayer at the transition from solid to liquid phase of the main bilayer lipid DPPC at 41.3 °C [11]. Mild hyperthermia (41-42 °C) could trigger almost complete drug release from LTSL within 20-40s at the melting temperature of 41.3 °C [67]. LTSL were proposed for application in a novel intravascular drug delivery approach in which liposomes are administered systemically while HT is being applied to the tumor, causing immediate drug release from the liposomes upon their arrival in the tumor area [68]. Thermodox has been used in two clinical studies – a Phase II trial in combination with local mild hyperthermia for patients with recurrent breast cancer of the chest wall, and in a Phase III trial combined with thermal ablation in patients with hepatocellular carcinoma [69, 70].

From these studies limited information is available yet, however adverse effects of LTSL and RFA in the treated patients were observed which resembled free drug side effects including; -neutropenia, leucopenia, lymphocytopenia, alopecia, fatigue, nausea, anorexia, fever. These toxicities are related to the heat-induced release in the tumor region in combination with the intrinsic instability of LTSL formulation causing large quantities of LTSL-released free drug in circulation [69-71]. LTSL in combination with hyperthermia in an intravascular release approach seems to be an attractive therapeutic option to improve doxorubicin delivery to tumors, but also leaves a room for further improvement by achieving more stable TSL formulations with rapid heat-triggered release and little to no premature drug release and related systemic toxicity.

Several other temperature sensitive liposomal formulations have been developed in the past decade that use different lipids and lipid compositions to increase drug release at elevated temperatures from intrinsically stable liposomes. Lindner et al. have incorporated phosphatidyl-oligoglycerols (DPPGOG) in their formulation (DPPG2-TSL) that contributes to prolonged circulation as well as to the thermosensitivity of the liposomal bilayer [63]. DPPGOG has a transition temperature of around 42 °C, which in combination with DPPC allows temperature controlled release of entrapped water-soluble molecules [63]. In presence of serum DPPG2-TSL proved more stable than LTSL [69]. Regarding release, different proteins in serum, such as albumin and immunoglobulin G, influenced liposome stability depending on lipid composition [69]. Also particle size appeared an important factor affecting doxorubicin release, as demonstrated for LTSL, which showed increased doxorubicin release at 37 °C with decreasing particle size. This

phenomenon was less prominent for DPPG2-TSL [72]. Another strategy to achieve improved stability with remaining high levels of content release from thermosensitive liposomes has been proposed by Li et al. [62]. In this study, lysolipid were omitted from the TSL, which were then further optimized with regard to lipid composition and density of DSPE-PEG_{2000}. Five mol% of DSPE-PEG_{2000} showed higher release kinetics than lower PEG-densities and this formulation released 60% of its content in 1 min at 42°C and 100% in 1h. The addition of more than 5 mol% of DSPE-PEG_{2000} destabilized the liposomal bilayer causing increased instability at 37 °C and lower encapsulation efficiency. Recently, this optimized PEG-TSL formulation proved more efficacious in the intravascular drug release approach in comparison to LTSL in a human BLM melanoma model [73]. Tagami et al. developed a LTSL related thermosensitive liposome formulation named HaT: Hyperthermia activated cytoToxic, in which the MSPC and DSPE-PEG2000 were replaced by with Brij78 (polyoxyethylene stearylether, 4 mol %) [25, 74-76]. The comparison between HaT and LTSL showed 1.2-2 fold faster release kinetics for HaT at 40-42 °C reaching 90-100% release in 2.5 min. In comparison with LTSL, HaT was able to deliver 1.4 fold higher levels of doxorubicin to the heated tumor (mouse mammary carcinoma). Additionally, the combination of HaT with HT significantly improved antitumor efficacy when compared to LTSL [74].

Although LTSL are at the forefront of pharmaceutical development and clinical application, and their application in the novel intravascular release approach holds promise, the intrinsic instability of this formulation and related risks for side effects are of concern. Novel formulations, lacking the lysolipid and with optimized densities of PEG-lipids, or other synthetic lipids such as DPPGOG or Brij-surfactants focus on further tailoring increased TSL stability in combination with (fast) heat-triggered release.

4. Intravascular and interstitial drug release.

When applying combinations of hyperthermia and TSL two different approaches can be followed. In the first one HT treatment is used to trigger interstitial drug release from liposomes that have accumulated in tumor tissue. The second approach triggers drug release intravascular from TSL that pass the heated tumor area.

Whereas the interstitial drug release approach relies on traditional EPR mediated accumulation of nanoparticles in the tumor area, the novel intravascular release approach is not dependent on this process of liposome extravasation [68]. Therefore, in the intravascular approach, heterogeneity in vessel permeability and nanoparticle penetration [7, 52, 77] is no longer an obstacle. Intravascular release of drugs from liposomes provides a continuous supply of drug to the tumor as long as the tumor is heated and drug-loaded liposomes pass the area. The established high intravascular drug concentrations in combination with hyperthermia increased interstitial fluid flow create drug gradients penetrating deeply into tumor tissue [68, 73]. An additional advantage is that in this approach the drug is readily bioavailable for both tumor and endothelial cells [68, 73, 78].

However, the intravascular drug release approach may suffer from drawbacks such as wash out of free drug from the tumor and related systemic side effects and requires precise heating of only the tumor area, which remains a challenge also in clinical practice. Therefore, although the intravascular release approach provides an efficacious delivery approach for drugs that are rapidly absorbed by tumor tissue in combination with precise tumor heating, the interstitial release approach, which relies on the content release from extravasated liposomes, can still be of significant value.

In that approach, hyperthermia can also be used prior to liposome injection to first increase tumor vascular permeability and subsequent liposome accumulation at the tumor. Several studies have shown that hyperthermia increased extravasation of liposomes in various mouse tumor models and in feline sarcoma patients [51, 52, 54, 55]. Alternatives to increase liposomal nanoparticle extravasation may also be used including the manipulation of tumor vasculature with biological agents increasing vascular permeability, e.g. TNF, IL-2 and Histamine causing more homogeneous nanoparticle and drug delivery [8, 79]. Upon achieving optimal liposome levels within the tumor interstitium, a next heat trigger can be applied to induce drug release in close proximity to tumor cells, facilitating intracellular drug delivery. In order to better understand which treatment option could ultimately be used clinically, additional studies are required evaluating both approaches. In such evaluation the tumor type, the possibility for precise local heating, and the type of drug used are important parameters that should be taken into consideration.

Image-guided drug delivery may provide an important tool in these studies and ultimately also in the clinic to monitor the localization of liposomes in tumors and their behavior.

5. Image-guided drug delivery

The possibility to monitor the intratumoral accumulation of liposomes during hyperthermia and subsequently their drug release would ultimately add significant benefit to the therapeutic outcome in the individual patient. Therefore, the development of non-invasive imaging techniques and imageable thermosensitive liposomes is of great clinical importance. Image–guided clinical intervention techniques using MRI, ultrasound or nuclear imaging such as X-ray computed tomography (CT), positron emission tomography (PET) and single-photon emission computed tomography (SPECT) are well established. PET and SPECT imaging are often used for visualization of liposome localization into certain types of tumors. For these purposes, liposomes can be labeled with radionuclides, from which the most often used are Tc-99m, In-111, Tl-201, F-18, I-123 and 131 and Ga-67 [2, 7, 55]. For the application of image-guidance in TSL-hyperthermia mediated drug delivery MRI has great potential. First of all it provides a good soft tissue signal and thus morphological/anatomical information and second MR thermometry could evaluate temperature alterations in different dimensions within the heated tissue [23, 27]. Thirdly, the hyperthermia-induced release of content can be monitored indirectly by co-release of an MRI contrast agent from the TSL. When inside the liposome, these contrast agents are not visualized due to the limited water

exchange across the bilayer. However, upon raising the temperature to the phase transition temperature of the bilayer, water exchange is increased and/or contrast agent is released from the liposomes thereby shortening the 1H MR relaxation time of the surrounding tissue [23]. Co-encapsulating both chemotherapeutic drugs and MR imaging agents in one carrier was demonstrated to provide a valid tool for image-guided temperature-induced drug release. Viglianti et al. and Ponce et al. e.g. have used doxorubicin and manganese as MRI contrast agent in one carrier. With this approach, in preclinical studies the concept of "dose painting" was demonstrated by MRI [26, 80]. However, manganese toxicity will limit the applicability of this approach [26, 80]. An improvement of this concept was achieved by Lindner et al. who used the clinically approved MR contrast agent Omniscan in DPPGOG-containing TSL and proved that, when HT is applied, the contrast agent is released and could be successfully monitored by MRI [24]. A similar approach was published by de Smet et al., who used the clinically approved MRI contrast agent Prohance co-entrapped with doxorubicin in traditional thermosensitive liposomes [27, 61]. They demonstrated the feasibility of this approach in HIFU mediated mild hyperthermia in a HIFU-MRI system [27]. The co-encapsulation of Prohance did not influence doxorubicin encapsulation or its release kinetics, whereas Prohance release at the transition temperature of the liposomes occurred simultaneously with doxorubicin [61]. Moreover, this contrast agent did not display any cellular toxicity. Recently, several studies have reported on MRI guided HIFU-mediated mild hyperthermia together with TSL in murine, rat and rabbit tumor models [25, 27, 28, 81]. In these studies, the combination of TSL with MRI guided HIFU hyperthermia resulted in higher doxorubicin concentrations delivered to the tumor when compared to TSL alone and free doxorubicin. Interestingly, the combination of LTSL with MR-HIFU hyperthermia in a rabbit tumor model showed increased doxorubicin delivery to the tumor core compared to LTSL alone and free doxorubicin treatment groups, of which drug delivery was limited to the tumor periphery [28]. Normally, drug delivery to the center of the tumor, an area with decreased perfusion and increased interstitial pressure, is limited. An explanation for this phenomenon is that the combination of LTSL and MRI guided HIFU caused increased perfusion, convection and vascular permeability in the tumor. All these findings demonstrate the feasibility of imaging-guided drug delivery as a useful approach for online monitoring the delivery of heat to the tumor and subsequent drug release from TSL and intratumoral accumulation, which helps to refine the treatment. As it has been shown that drug delivery efficiency depends on the tumor type and morphological characteristics such as vascularization, necrosis and permeability, image-guided drug delivery may serve as a powerful tool to personalize hyperthermia-mediated TSL-chemotherapy for cancer.

6. Targeted liposomes

A second approach to improve drug bioavailability in combination with a further improvement in tumor (cell) selective intracellular drug delivery may come from active targeting of liposomes to the tumor. This can be achieved by decorating liposomes with targeting ligands specific for receptors uniquely or overexpressed on the target cells. By

doing so, this may result in higher levels of retention of liposomes at the tumor site, cell specific binding and drug delivery [2, 82-84]. In addition, upon targeting an internalizing antigen, the ligand can induce receptor-mediated endocytosis of the liposomal nanocarrier, which upon intracellular processing may release the entrapped drugs [2, 83]. Intracellularly released drug will be prevented from rapid diffusion from the tumor area and will be delivered across the cell membrane barrier close to its active site, which for most chemotherapeutic drugs is intracellular; in the cytosol or the nucleus. Via these ways targeting of liposomes may contribute to increased therapeutic efficacy.

Regarding the targeting ligand, a careful selection is required, concerning its selective expression or overexpression on the target cell, possible shedding of the targeting ligand and its capacity for receptor-mediated endocytosis [85-89]. Several targeting ligands have been studied for development of targeted liposomes, including antibodies and their fragments, peptides, vitamins, carbohydrates, nucleic acids and charged lipids. It is important that liposomal attachment of targeting ligands does not interfere with normal physicochemical characteristics of the liposomes and the targeting moiety such as size, stability, drug retention and receptor-binding affinity nor affects liposome pharmacokinetics, biodistribution and tumor accumulation [2, 83, 90, 91.]

Antibody targeting is the most commonly used and extensively studied means of targeting liposomes with encapsulated chemotherapeutic drugs. This is related to the usually high affinity of antibodies for their targets. Additionally, several monoclonal antibodies that have reached clinical application display therapeutic activity by themselves which can contribute to increased therapeutic activity, e.g. trastuzumab (Herceptin) targeting HER2 receptors overexpressed on some types of breast cancer [92], bevacizumab specific for the VEGF receptor which is in use as an inhibitor of angiogenesis [84, 92].

For liposome targeting, basically two main approaches can be followed that are distinguished based on the target cell type; 1. vascular targeting, 2. tumor cell targeting. Vascular targeting does not rely on nanoparticle extravasation and has the advantage that circulating liposomes have direct access to tumor endothelial cells. Vasculature-targeted liposomes often utilize proteins overexpressed on angiogenic endothelial cell membranes, such as integrins, adhesion molecules or growth factor receptors [93]. Binding through these receptors may lead to liposome internalization by endothelial cells and subsequent drug release, which can thereafter induce endothelial cell death, vascular damage and subsequent massive tumor cell death.

The second approach of tumor cell targeting relies on liposome extravasation into the tumor interstitium, where the targeting ligand can specifically recognize and bind the target receptor. Upon tumor cell binding, targeted liposomal chemotherapeutics have been demonstrated to improve therapeutic efficacy over non-targeted liposomes [94-96].

6.1. Tumor vasculature targeting.

Angiogenesis, the formation of new blood vessels from preexisting ones is a crucial process in tumor growth and metastasis. Targeting of tumor vasculature to

interrupt this process has become an attractive approach to suppress tumor growth because of the important role tumor vessels play in tumor pathophysiology by providing nutrients and oxygen to the proliferating tumor cells. Hence, destroying endothelial cells in tumor vessels may affect a multitude of tumor cells. Tumor vasculature targeting can be achieved by the cell surface expression of angiogenesis-related receptors on the endothelial cells and their direct accessibility for circulating targeted liposomal therapeutics [97]. Therefore, in contrast to tumor-cell targeted liposomes, they do not need to cross the endothelial cell layer and are not hampered by the relatively high intratumoral fluid pressure, which may prevent deep tumor penetration [98]. Specific targets on tumor vasculature include αvß3 integrins [99], receptors for angiogenic growth factors (such as VEGFR, EGFR) [84], aminopeptidase N (e.g. CD13) [100] and the overexpression of negatively charged molecules [98].

An extensively studied target for tumor vasculature-targeted liposomes is the VEGF receptor. VEGFR2 has been found upregulated on angiogenic endothelial cells [92] and bevacizumab therapy has been developed for blocking VEGFR receptor function and thus angiogenesis. Although antibody therapy alone is efficacious in cancer treatment [92], their attachment to liposomes offers additional advantages. Immunoliposomes having both a specific antibody for tumor vasculature and a chemotherapeutic drug, which may have an additive or synergistic effect, may eventually have an increased therapeutic potential [101]. Wicki et al. have shown that anti-VEGFR2 immunoliposomes loaded with doxorubicin had an increased therapeutic efficacy when compared to antibodies alone in a Rip1Tag2 mouse model for insulinoma, in MMTV-PyMT mouse model for breast cancer, and in a HT-29 human colon cancer xenograft transplantation model [84]. Additionally, anti-VEGFR2 immunoliposomes were much more effective in reducing tumor growth compared to non-targeted pegylated liposomal doxorubicin [84]. Several tumor vasculature specific peptides have been used for liposome targeting. The NGR peptide has been proven to interact selectively with aminopeptidase N (APN) isoforms (CD13) overexpressed on tumor vasculature and not on normal vasculature [100]. Pastorino et al supported the idea that due to the internalizing properties of NGR epitope, liposomes after binding and internalization are degraded by lysosomal or endosomal enzymes, causing intracellular drug release and cytotoxicity. NGR targeted liposomes proved effective in reducing tumor vascularization and tumor growth as shown in an orthotopic neuroblastoma mouse model [100]. More recently Loi et al. have shown that mice injected with aminopeptidase A (APA)-targeted Dox-containing liposomes, targeting perivascular cells, had a prolonged life span than control mice but shorter than mice treated with APN targeted Dox liposomes. However, the combination therapy of APN- and APA-targeted Dox-containing liposomes led to the largest increase in life span compared to each of the individual treatments. Moreover, the combination treatment was able to cause massive destruction of tumor vasculature with depletion of endothelial cells and the pericytes [102]. Another study by Moura et al. demonstrated that the F3-peptide targeted liposomes (binding to NH2 terminal domain of nuclein) were able to target both tumor and endothelial cells in human orthotopic tumors, implanted in the

mammary fat pad of nude mice [103]. RGD-targeted liposomes represent another interesting vasculature-targeted liposome formulation directed to αvß3 integrins overexpressed on angiogenic tumor vasculature. In several studies it has been demonstrated that RGD-targeted liposomes are specific for proliferating tumor vasculature and are able to decrease tumor growth in comparison with non-targeted formulations [99, 104, 105]. An alternative way of targeting tumor vasculature involves the use of cationic lipids incorporated in the liposomal bilayer for specific interaction with anionic molecules overexpressed on angiogenic endothelial cell membranes [106, 107]. These anionic molecules are part of the glycocalyx of the endothelial lining: glycoproteins, glycolipids, proteoglycans whose distribution on blood vessels is patchy and heterogeneous. In addition, the sluggish and irregular blood flow in tumors facilitates the selective targeting of cationic liposomes to angiogenic endothelial cells. Alternatively, Ho et al. have shown that cationic liposomes can be opsonized by yet unidentified proteins inducing specific binding to angiogenic endothelial cells . Images in figure 2 represent an example of tumor vasculature targeting of cationic liposomes described in Dicheva et al. 2013 . In this study vasculature was visualized in green by the constitutive expression of green fluorescent protein (GFP) in endothelial cells. Cationic liposomes (in red) can be seen as immobile patchy fluorescent hotspots attached to the vessel wall. In several studies it was shown that cationic liposomes loaded with chemotherapeutic drugs were able to diminish tumor growth and prolong survival when compared to control non-cationic formulations [107, 110-112].

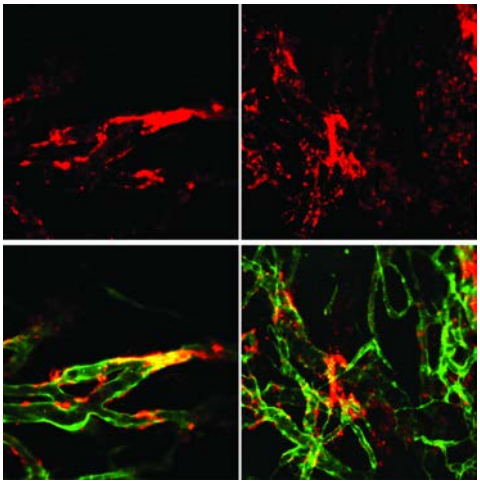

Fig 2. Binding of cationic thermosensitive liposomes to angiogenic tumor vasculature of B16BL6 window chamber bearing mice 24h after liposome injection. Liposomes were injected at a concentration of 5 μmol lipid and were labeled with rhodamine-PE (red). Tumor vasculature is visualized in green due to the expression of GFP protein in the endothelial cells. Unpublished observation from a study by Dicheva et al [19].

In conclusion, the use of liposomal therapeutics targeted to tumor vasculature represents a promising approach in cancer therapy. The angiogenic tumor endothelium offers easy access and opportunities for specific recognition for circulating vasculature-targeted liposomes. The direct attack on tumor vasculature and induction of vascular damage will affect tumor cells that strongly depend on the supply of oxygen and nutrients. In this way the cytotoxic action targeted towards endothelial cells will induce death of a multitude of underlying tumor cells.

6.2. Tumor cell targeting.

Next to vascular targeting, liposomes can be designed for tumor cell targeting. An important requirement for this approach is extravasation of nanoparticles through the angiogenic tumor endothelium, a process which requires small size and prolonged presence of liposomes in systemic circulation. Upon extravasation, liposomes come in close proximity of tumor cells allowing the targeting ligand to bind to tumor cell specific receptors. The binding will increase the retention of the nanoparticle in the tumor and prevent wash out of liposomes from the tumor to the circulation. Ligand-specific binding may also promote internalization. Strategies for tumor cell targeting have been developed involving both antibodies and peptides specific for tumor cell surface markers. For instance, targeting of HER2 overexpressing breast cancer has been achieved using anti-HER2 immunoliposomes. A study by Park et al. demonstrated that anti-HER2 immunoliposomes displayed significant therapeutic efficacy in four different HER2 overexpressing breast xenograft models. Anti-HER2 immunoliposomes were much more efficient in reducing tumor growth than free doxorubicin, non-targeted liposomal doxorubicin or anti-HER2 MAb (trastuzumab) [13]. This study demonstrated that conjugation of a drug-loaded liposome to a therapeutic antibody can exert dual antitumor activity. Another potential receptor for targeted liposomes, which is overexpressed on tumor cells, is the EGF receptor. Mamot et al. developed EGFR-targeted immunoliposomes and reported higher cellular toxicity of the encapsulated drugs by EGFR-targeted immunoliposomes compared to non-targeted liposomal drug in EGFR-overexpressing cancer cells, thereby demonstrating the importance of active intracellular drug delivery [113]. In addition, superior tumor growth inhibition was demonstrated for these immunoliposomes when compared to non-targeted liposomes or free drug [114]. An important development is the clinical application of EGFR-targeted doxorubicin liposomes in a phase 1 trial. Results from this study demonstrated the treatment was tolerable and warrants further testing of this targeted liposomal nanomedicine in a phase II trial [115]. It is important for tumor cell targeting that the targeting ligands do not interfere with liposome pharmacokinetics and biodistribution and in that sense promote optimal liposome extravasation similar to non-targeted liposomes. When using whole antibodies, exposure of the Fc-domain may interfere with liposome circulation and thus tumor accumulation [90, 91]. In order to diminish fast clearance, limited antibody density should be chosen or the Fc-domain should be abandoned completely by using antibody fragments (Fab'2, Fab', scFv, or single domain variable fragments) attached to the liposomes. Griffiths et al. have shown a

superior amount of doxorubicin delivered to tumor cells by Fab-targeted liposomes in comparison with non-targeted liposomes and these liposomes hold promise for in vivo application [116]. In another study by Pastorino et al., mice with neuroblastoma, treated with doxorubicin-liposomes targeted with Fab' specific for disialoganglioside GD2 overexpressed on these tumors, exhibited prolonged survival over mice treated with non-targeted Dox-liposomes or free doxorubicin [117]. Allen et al. demonstrated that internalization of targeted liposomal chemotherapy is of crucial importance for efficacy, by showing that doxorubicin loaded internalizing anti-CD19-targeted liposomes caused improved survival in lymphoma bearing mice compared to those injected with non-targeted or anti-CD20-targeted non-internalizing liposomes [89]. In addition to vasculature targeting, tumor cell targeting can provide a powerful tool to increase the efficacy of chemotherapy due to the specific uptake of liposomes in tumor cells. However, unlike vascular targeting, tumor cell targeting may require various specific antibodies and liposomal conjugates for different tumor types and even tumor type subclasses as they will greatly differ in the expression of biomarkers. This will limit the development of a general antibody targeted treatment approach for large groups of tumors, but will require the availability of a multitude of targeted formulations and also screening of patients for biomarker expression. An additional disadvantage could be the so-called binding site barrier phenomenon as described for monoclonal antibodies by Weinstein et al. [118]. Tumor cell targeted immunoliposomes upon extravasation may bind immediately to the first receptorpositive tumor cells encountered, being those located perivascularly, thereby preventing liposomes and drug from further penetration into the tumor tissue. The use of whole antibodies in this respect may, also upon extravasation in the tumor, promote Fc-receptor mediated uptake by tumor associated macrophages, preventing efficient tumor cell drug delivery [90,91]. Therefore, the use of antibody fragments may not only prevent rapid uptake of liposomes by tissue macrophages in liver and spleen, but also aid to escape Fc-receptor mediated endocytosis by macrophages present in perivascular tumor regions and promote tumor cell specificity [116, 117].

Tumor cell targeted liposomal chemotherapeutics have demonstrated potential to improve tumor specific drug delivery and improved bioavailability in preclinical studies. Further studies will now have to demonstrate clinical benefit. It is encouraging that such studies were initiated recently. Despite the specific delivery and the higher uptake of liposomes in tumor cells due to internalization, there is still room for improvement of this targeted drug delivery concept. Usually liposomes are slowly degraded by tumor cells resulting in suboptimal bioavailable drug concentrations [8]. Therefore, application of an external trigger, e.g. heat, can be an important means to achieve intracellularly triggered drug release to improve and control drug bioavailability from targeted liposomes [62, 63]. Such a heat trigger will in addition also improve extravasation of tumor cell targeted nanoparticles [73]. Therefore, the combination of heat with liposomes that are targeted and thermosensitive may help to further improve liposomal chemotherapy.

7. Targeted thermosensitive liposomes.

Despite the various approaches developed to improve tumor drug delivery and drug bioavailability such as improving nanoparticle extravasation, triggered release and cell specific targeting, still further improvement could be achieved, for instance from smart combinations of these individual approaches. Therefore, recent progress in the development of targeted liposomes with heat-induced delivery and drug release properties will be reviewed in this section. Such targeted and triggerable systems can improve tumor retention through interactions with cells, target the nanoparticles specifically and can be triggered for drug release upon intracellular arrival, all contributing to improved drug bioavailability and thus efficacy. From the point of view of the TSL, a combination with targeting can help to improve TSL tumor retention, provide cell specific drug delivery and intracellular drug distribution upon heat. From the point of view of the targeted liposomes, designing them with a thermosensitive bilayer and apply them with a heat trigger may enhance tumor accumulation and trigger intracellular drug release close to their molecular targets. Although several different strategies can be applied to improve drug bioavailability from targeted and internalized liposomal nanocarriers, e.g. using pH dependent fusogenic peptides [119] or lipids [120], light-sensitive probes enhancing endolysosomal escape [14], we here focus on the use of heat and thermosensitive liposomal bilayers in combination with cell-specific targeting.

Targeted TSL (TTSL) will require the attachment of specific ligands like peptides and antibodies to the liposome or the incorporation of cationic lipids in their bilayer (figure 3).

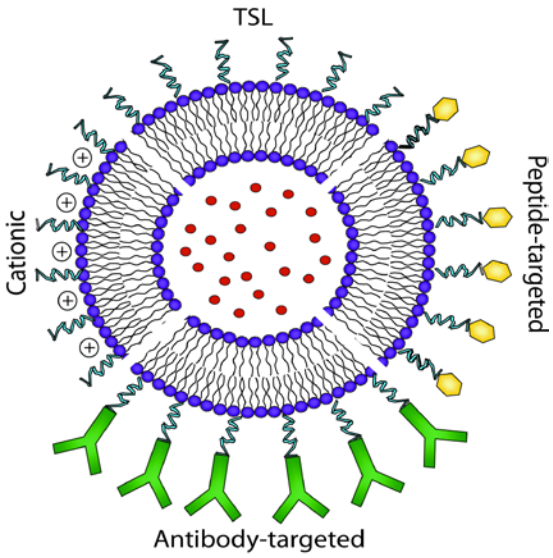

Fig 3. Schematic representation of three different approaches to target thermosensitive liposomes. Peptides attached to PEG (right), antibody conjugated to PEG (bottom) and cationic lipids in the lipid bilayer (left).

When TSL are targeted to the tumor vasculature, the intravascular release approach may be exploited more optimal for delivery of high quantity of chemotherapeutic drugs in the tumor area. For example, the current approach for intravascular drug release requires ultra-fast releasing TSL formulations, which unfortunately are characterized by intrinsic instability and risks for increased free drug toxicity. As an alternative, more stable TTSL could be targeted to tumor vasculature from which heat- triggered release may render control over drug release and in combination with the improved stability cause less premature drug release. In such an approach one needs to determine the optimal sequence of hyperthermia and nanoparticle administration to achieve maximal tumor accumulation, either at the vasculature, or after extravasation at the level of the tumor cells and efficient HT triggered drug release. Tumor vasculature targeted TSL will be bound to or internalized by angiogenic endothelial cells from where a heat trigger can release the drug extra-or intracellularly (figure 4).

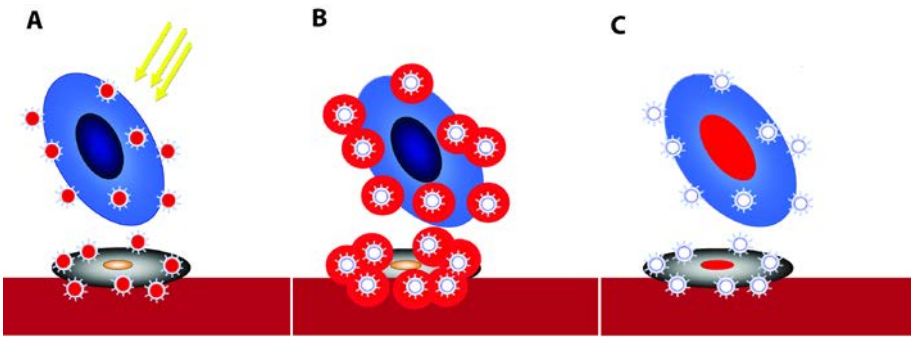

Fig 4. Schematic representation of targeted thermosensitive liposomes bound to tumor cells and endothelial cells (A), triggered release (B) and nuclear drug uptake (C).

From these intravascular or intracellular release sites drug may effect endothelium and upon penetration into tumor tissue, also tumor cells. TTSL may have additional benefit by targeting yet unidentified micrometastases. In comparison with non-targeted liposomes, TTSL, due to their high affinity for tumor vasculature or tumor cells may reach and specifically accumulate into these micrometastastic nodules and in a targeted way deliver their drug contents passively into these tumors. Upon equipping these TTSL with contrast agents, they may aid visualization of metastatic loci and subsequent local heating, e.g. by using HIFU to trigger drug release.

Although literature on targeted thermosensitive liposomes is limited, several groups have started to explore this novel strategy of targeted temperature-triggered drug delivery (Table 1).

Table 1. Overview of targeted thermosensitive liposomes applied with external hyperthermia.

Formulation	Cell surface antigen	Targeting ligand	Liposome composition and molar ratio*	Content*	Tumor model (vitro/vivo)	Reference
Folate-TSL	Folate receptor	Folate	DPPC:HSPC:Chol:PEG-PE:Folate-PEG-PE 53: 27: 16: 3: 1	doxorubicin	Cervical carcinoma (vitro)	Gaber 2002[15]
HER2-TSL	HER2	anti-Her2 affibody	DPPC:Mal-PEG_{2000}-DSPE: PEG_{2000}-DSPE 89: 5.5: 5.5	Calcein, DiR, Rho-PE, doxorubicin	Breast carcinoma (vitro)	Puri 2008[18] Smith 2011[21]
two component HER2-TSL	HER2	anti-HER2 antibody Trastuzumab	1. DPPC:MPPC: DPPG: PEG_{2000}-DSPE-:NHS-PEG_{3400}-DSPE- 82:7: 4: 4: 4 2. DPPC:MPPC:RhoPE: NHS-PEG_{3400}-DSPE 86:7:3: 4	1. Calcein 2. Rho-PE	Breast carcinoma (vitro)	Kullberg 2009[16]
HER2-LLO-TSL	HER2	anti-HER2 antibody Trastuzumab	DPPC/MPPC/DPPG/NHS- PEG_{3400}-DSPE 82:10: 3.5: 4	Listeriolysin, calcein	Breast carcinoma (vitro)	Kullberg 2010[17]
NGR-LTSL	CD13	Cyclic NGR peptide	DPPC:MSPC:NGR-PEG_{2000}-DSPE 85:10: 5	DiO, doxorubicin	Fibrosarcoma, Breast carcinoma (vitro)	Negussie 2010[20]
Cationic TSL	Anionic molecules on tumor vasculature and tumor cells	Cationic lipid	DPPC: DSPC: PEG_{2000}-DSPE: DPTAP 60: 25: 5: 10	Rho-PE, CF	Endothelial cells Melanoma Lung carcinoma (vitro and vivo)	Dicheva 2012[19]

In 2002, Gaber et al. described the first TTSL formulation in a paper on folate-targeted TSL[15]. These TTSL loaded with doxorubicin were able to circumvent multidrug resistance through folate receptor mediated liposome internalization and hyperthermia triggered intracellular drug release. More recently, Puri et al. and Smith et al. developed affibody-conjugated thermosensitive liposomes specific for HER2 positive tumor cells [18, 111]. They demonstrated 10-fold increased specificity of these TSL in vitro in comparison with non-targeted liposomes in HER2 positive cells and a lack of binding to HER2 negative cells. Additionally, hyperthermia at 45 °C for 20 min caused substantial release of a liposome encapsulated marker in the cytosol. Similar TTSL, loaded with doxorubicin were able to deliver 2-3 fold more doxorubicin into HER2 positive cells in comparison with non-targeted TSL. Therefore, these liposomes combining both targeting and triggered release properties in one carrier hold promise for improved efficacy in breast cancer treatment [111]. A drawback of targeted liposomes may be their eventual uptake by acidic compartments in cytosol (endosomes, lysosomes) where the low pH may hinder the escape of the heat- released drug from these

organelles. Kullberg et al. indeed demonstrated the problem of endo/lysosomal escape of TSL released contents [16]. However, they also presented a potential solution for this problem by proposing the use of a pore-forming molecule. TTSL were equipped with lysteriolysin O, which is able to create pores in the endosomal membrane, thus allowing the released liposomal content in the endosomes to reach the cytosol [17]. In this study, TTSL targeted to HER2 overexpressing mammary epithelial cells were able to deliver 22-fold higher amount of the encapsulated liposome marker (calcein) into HER2 positive cells than to HER2 negative ones. In vivo studies accessing efficacy and possible toxicity to normal cells of this advanced drug delivery approach is not yet available.

Besides tumor cell targeting some groups also addressed tumor vasculature targeting of TSL. Dreher et al. have designed thermosensitive liposomes targeted to tumor vasculature through cyclic NGR peptides exposed on the liposomal surface [20]. These liposomes showed a potential for improving chemotherapy due to their high specificity for CD13+ cells in vitro and demonstrated clear temperature-release kinetics when hyperthermia was applied. Recently, we developed another novel targeted thermosensitive liposome formulation containing cationic lipids in their bilayer [19]. Similar to Dreher's approach, our cationic TSL displayed specific binding to angiogenic endothelial cells, but in addition also to tumor cells in vitro and in vivo. Moreover, these dual-targeted liposomes were stable at physiological conditions and released their content extra-and intracellularly under hyperthermia both in vitro and in vivo [19].

In a related field of magnetic targeting and alternating magnetic field (AMF)-induced hyperthermia several additional examples of triggering content release from targeted liposomes are available. The difference here is that the development of heat occurs in the liposome itself through interaction of the AMF with the entrapped magnetic nanoparticles causing heat [121]. Pradhan et al. reported on the development of both biologically (by a folate ligand) and physically (by a magnet) targeted TSL [122]. These liposomes yielded an increase in doxorubicin uptake as compared to Doxil or non-magnetic folate targeted liposomes in cells overexpressing a folate receptor. Additionally, magnetic hyperthermia was able to increase cellular toxicity of these liposomes. Kikumori et al. targeted in vivo magnetoliposomes to HER2-positive breast cancer cells and proved a significant tumor regression in the AMF-hyperthermia treated group [123]. Therefore, targeted magnetoliposomes in combination with AMF-induced hyperthermia may also provide a powerful tool in cancer treatment.

In conclusion, the field of TTSL in combination with HT triggered drug release is underexplored thus far, however promising achievements are emerging. Yet, firm in vivo proof of efficacy of this approach proving the benefits of TTSL is scarce and should be a focus for near future studies. Especially in combination with imageable TTSL this approach could be further optimized by image-guidance through visualization of tumor specific targeting and identification of possible micrometastases which could next be heated to achieve rapid and maximal drug delivery.

Conclusion. The application of targeting and of heat-triggered drug release from thermosensitive liposomes represent further improvements of conventional liposomal chemotherapy. Targeted liposomes benefit from high specificity for tumor cells and/or tumor vasculature, which will increase their retention in tumors and promote liposome internalization and intracellular drug delivery. Thermosensitive nanoparticles combined with hyperthermia can also help to overcome the low drug bioavailability of conventional liposomes by improved tumor accumulation and triggered drug release from the nanoparticles. When combining the increased liposome specificity from targeted liposomes with temperature sensitive bilayers and hyperthermia, further improvement of intracellular drug delivery can be achieved. To achieve this, various tools are available, that should be applied in an optimal way to guarantee efficient drug delivery. Such combinations together with image-guided drug delivery have potential to improve the therapeutic outcome of liposomal chemotherapy for cancer and warrants further personalization of cancer chemotherapy.

Expert opinion. Liposomal encapsulation has improved cancer chemotherapy in the past decade. Stable drug entrapment in small (<100 nm) long-circulating liposomes has contributed significantly to improved treatment of cancer patients. Up to now clinical outcome demonstrates major benefit of this approach to decrease toxicity of chemotherapy. Although therapeutic activity has been clearly observed in various tumor types, in general no strong improvement was observed compared to free drug treatment. This can be explained by several factors including limited tumor accumulation and specificity of liposomal nanoparticles, low bioavailability of drug contents due to (too) stable entrapment and lack of control of drug release. Many novel approaches have been proposed in the past years to tackle these issues and thereby improve liposomal chemotherapy. Two key approaches for improvement are the subject of this review being cell-specific targeting and temperature-controlled drug release.

Cell specific targeting of liposomal nanoparticles can benefit tumor specificity of drug delivery by recognition of cell surface moieties that distinguish tumor vasculature or tumor cells from normal tissues. Targeting tumor vasculature overcomes the important hurdle of nanoparticle extravasation into tumor tissue and can potentially affect a multitude of tumor cells upon drug delivery to and damaging of single tumor vessels. Important requirements are the targeting of tumor vasculature specifically, using angiogenesis-specific receptors, and delivery of drugs able to effectively impinge on endothelial cells. Tumor cell targeted nanoparticles require extravasation across the tumor vascular endothelium in order to access tumor cell specific receptors for binding. Such nanoparticles in principle do not promote increased total tumor drug delivery levels, but rather the precision and intracellular localization of the chemotherapy. Specific ligands are available as well as conjugation strategies for nanoparticle assembly to allow for the targeted approach and preclinical studies have demonstrated therapeutic benefit. Yet, clinical application employing targeted liposomal

chemotherapy has been initiated and will hopefully demonstrate therapeutic benefit of the targeting.

Hyperthermia to improve tumor accumulation of nanoparticles and control drug release from thermosensitive liposomes provides an attractive therapeutic option to improve liposomal chemotherapy by overcoming the tumor vascular barrier and providing control of intratumoral delivery of bioavailable drug from the nanoparticles. Several thermosensitive liposomal formulations demonstrated improved therapeutic activity compared to doxorubicin or non-thermosensitive doxorubicin liposomes in preclinical setting. Triggered drug bioavailability can be achieved by two different strategies; interstitial and intravascular drug release. The interstitial release approach relies on hyperthermia-enhanced liposome extravasation and tumor accumulation followed by triggered interstitial drug release in close proximity of the tumor cells. The novel intravascular drug delivery approach uses tumor specific heating to trigger drug release from circulating thermosensitive liposomes in the tumor vasculature, thereby inducing tumor drug influx along the created drug gradient from the high concentration in tumor vasculature into the tumor tissue. This promising approach requires very precise heating of the tumor and fast release from the nanoparticle in combination with drugs that are rapidly taken up by tumor vasculature and/or tumor cells. The application of lyso-PC based thermosensitive liposomal doxorubicin with hyperthermia or radiofrequency ablation is currently being evaluated in clinical trials, which is an important development. Although conclusions on the outcome of these studies are not available yet, improvements on thermosensitive liposome design have been proposed to improve liposomal drug retention in circulation in combination with effective and intratumorally triggered drug release. In addition, the application of imaging and liposomal encapsulation of contrast agents has been developed to allow for online monitoring of tumor delivery of both heat and drugs through image-guidance and will benefit further personalization of chemotherapy delivery.

Combinations of both the targeted and temperature triggered drug delivery approaches have been described and can help to further improve tumor specific chemotherapy delivery by several means. In case of intravascular drug release from thermosensitive liposomes an increased retention or prolonged presence of the nanoparticles in tumor vasculature through promoting tumor vascular interaction, could ensure improved and more specific tumor drug delivery. Next, hyperthermia can increase tumor vascular permeability and promote extravasation of tumor cell targeted nanoparticles and their interaction with tumor target cells. A temperature shock may further promote drug bioavailability from tumor cell targeted liposomes either extra- or intracellularly. Moreover, targeted systems may aid identification of distant metastases and promote heat triggered drug delivery at these sites upon image- guidance.

Up to now only a limited number of studies focused on the targeted thermosensitive drug delivery approach. Near future in vivo studies will have to demonstrate the therapeutic potential of this novel strategy.

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Chapter 7

General discussion

Discussion

One of the crucial problems with current cancer chemotherapy is related to a small therapeutic window because of its toxicity to vital organs such as bone marrow, heart, liver, kidney and mucosal surfaces. This toxicity in general does not allow for optimal dosing to obtain important tumor responses. Moreover certain pathophysiologic characteristics of tumors further impede sufficient intratumoral drug delivery. The research described in this thesis was dedicated towards improving chemotherapy. This can be a reason for relapse of the tumor and the appearance of drug resistance [1]. The medical research community has searched for therapies which target specifically tumor cells while sparing normal cells. Although remarkable progress has been made in cancer therapy, many cancers are still little or nonresponsive to these conventional therapies. Therefore, new modalities to improve chemotherapy, that can increase therapeutic efficacy, need to be investigated. In this thesis, liposomes were chosen as a means to increase therapeutic indexes of anticancer drugs. The aim was to locally deliver chemotherapeutic drugs, such as doxorubicin to the tumor in order to increase efficacy and to reduce side effect to normal tissues (Fig 1). Although liposomes have been proven successful in several tumor types, their efficacy is often not better to the free drug. Progression-free survival and overall survival of patients treated with pegylated liposomal doxorubicin (PLD) and free doxorubicin remain similar [2]. However the benefits are associated with a decrease of toxicities: PLD decreases cardiotoxicity, myelosuppression, alopecia and vomiting. Reasons for the lack of strong improvement of liposomal chemotherapy [2-4] is the non-specific nature of liposomes leading to limited tumor accumulation [5] and their high intrinsic stability [6-8] causing low drug bioavailability. Liposomes have to circulate for days in order to reach the tumor in high concentrations [9]. Liposome accumulation in the tumor tissue competes with their accumulation in liver and spleen and only 10% or less of the injected dose will eventually reach the tumor [5]. Doxil has long circulation due to its stability and ability to escape clearance by the RES. However, due to its stability the doxorubicin bioavailability is low [8]. Seynhaeve et al. demonstrated that Doxil is taken up intracellularly in lysosomes, where it is retained intact for a relatively long period, which impairs delivery of doxorubicin to the nucleus [10]. Therefore, a drug delivery system which possesses specific affinity for the tumor and ability to release its drug payload at the tumor site, is needed. For this purpose, we created targeted thermosensitive liposomes (TTSL), which incorporate both the targeting and triggered functions of a liposome in one carrier. In this way, both low tumor retention and low drug bioavailability can be circumvented.

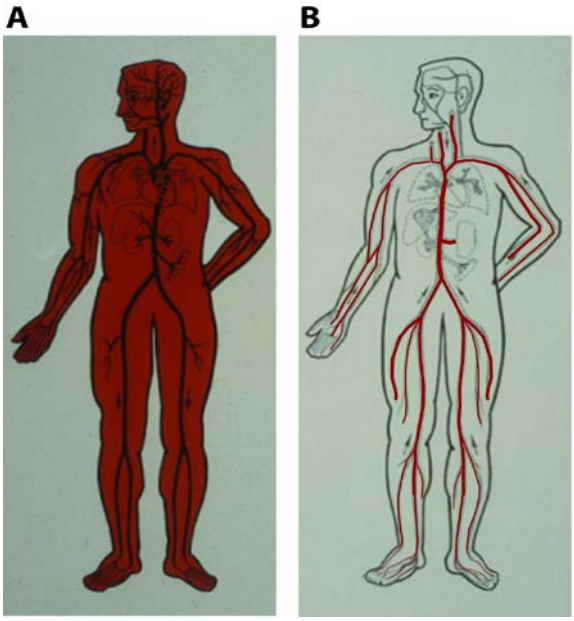

Fig 1. Theoretic distribution of a free drug (A) and liposomal drug (B) throughout the body, emphasizing the difference in volume of distribution.

Liposome formulations

In order to increase liposomes retention in the tumor and drug bioavailability, the liposomes used in the presented work were both targeted and thermosensitive. They contained in their lipid bilayer thermosensitive lipids, which upon HT were able to translocate and create permeable areas in the liposomal membrane for transition of the entrapped drugs through it. Thus, HT applied locally to the tumors actively triggered drug release from TSL. Once released in the tumor area, the free drug can cross the cell membranes and reach its site of action, the nucleus. Additionally, to further improve drug delivery, our liposomes were targeted. When liposomes are targeted, they can recognize specific receptors either on tumor or endothelial cells, which can promote receptor-mediated endocytosis. Thereafter, the endocytosed liposomes may release intracellularly the encapsulated drug, favoring transport to the nucleus. Cellular targeting is due to the specific affinity of liposomes decorated with molecules recognizing cell surface moieties distinguishing tumor cells from normal cells. Using these improvements of liposomes, we aimed at increasing therapeutic efficacy of liposomal chemotherapy. In the studies described we followed two main ways of liposome targeting based on different cell types: tumor cell targeting and vascular endothelial cell targeting. Endothelial cell targeting has the advantage that liposomes do not need to extravasate through tumor vasculature and endothelial cells are easily accessed by liposomes. This targeting can contribute to killing of millions of tumor cells relying on oxygen and nutrients derived from tumor vasculature. On another hand, tumor cell targeting relies on extravasation of small and long circulating liposomes that

will eventually reach tumor cells. The close localization of liposomes to tumor cells will allow binding between the targeting ligand and cell-specific receptors. This specific binding prevents the liposomes from wash out from the tumor to the circulation. In comparison to vascular targeting, tumor cell targeting involves the use of different targeting ligands specific for tumor cell receptors in the different tumor types expressing different biomarkers. Therefore, this targeting approach cannot depend on a unique molecule attached to the liposome, which limits its application. Another disadvantage of tumor cell targeting is the so-called binding site barrier phenomenon [11]. Tumor cell targeted immunoliposomes may bind immediately to the first receptor-positive tumor cells encountered upon extravasation, being those located perivascularly, thereby preventing liposomes and drug from further penetration into the tumor tissue. The use of whole antibodies in this respect may, also upon extravasation in the tumor, promote Fc-receptor mediated uptake by tumor associated macrophages, preventing efficient tumor cell drug delivery [12, 13]. Therefore, the use of antibody fragments may not only prevent rapid uptake of liposomes by tissue macrophages in liver and spleen, but also aid to escape Fc-receptor mediated endocytosis by macrophages present in perivascular tumor regions and promote tumor cell specificity [14, 15]. Anti-HER2 immunoliposomes have demonstrated significant antitumor efficacy in several tumor models when compared to nontargeted immunoliposomes [16]. Mamot et al. have reported on a significant tumor growth inhibition by EGFR-expressing immunoliposomes compared to nontargeted immunoliposomes [17]. Tumor cell targeting has achieved promising results in cancer treatment but there is still a room for improvement. Despite its advantages, this targeting suffers from the fact that once liposomes reach and bind specifically to tumor cells, drug release is slow and inefficient, which limits its therapeutic efficacy. Therefore, the combination of targeted and triggered release features of liposomes in one carrier may help to overcome some of the drawbacks of currently available liposomal chemotherapy. This recently emerging field of targeted thermosensitive liposomes (TTSL) in cancer chemotherapy will benefit from the increased tumor retention of the carrier together with triggering drug release upon its arrival in the tumor. Several approaches are available for enhancing drug bioavailability. In the presented work, we focus on the use of HT and thermosensitive liposomes in combination with cell-specific targeting. Although the field of TTSL has already showed promising results, there is still more to be done concerning its therapeutic efficacy [18-26] . In this thesis, two targeted thermosensitive liposome formulations were investigated; cationic thermosensitive liposomes (CTSL in Chapter 2 and 3) and RGD-targeted thermosensitive liposomes (RGD-TSL in Chapter 4). These two formulations differed in the targeting ligands. In the case of CTSL, cationic molecules were incorporated in the liposome bilayer for specific binding to overexpressed anionic sites on the endothelial or tumor cell membranes. RGD-TSL made use of RGD peptides attached to the lipid bilayers for recognition of αvβ3 integrins overexpressed on endothelial cells and some types of tumor cells. Both formulations showed stability at physiological temperatures of 37 °C and slightly started to release their drug payload at 39 °C. The observed release kinetics are in accordance with non-targeted

thermosensitive liposomes proven successful for in vitro and in vivo heat-triggered content release [27] . Maximum drug release of our TTSL occurred at temperatures between 41-43 °C, which are clinically tolerable temperatures locoregionally and in 1h they released almost 100% of their content, time for heating which can be applied in clinics. Besides their temperature sensitive properties, both CTSL and RGD-TSL could bind specifically to tumor and endothelial cells. Upon binding, the targeted liposomes were internalized into the acidic cellular organelles. Using lysotracker as a lysosomal marker, we could prove that a big part of both CTSL and RGD-TSL are internalized into the lysosomes (Chapter 3 and 4). The applied HT triggered drug release into lysosomes. The released Dox there could have difficulties to escape the lysosomes because of the low pH in these organelles, which may cause precipitation of the drug [28]. An interesting way to stimulate Dox release from acidic compartments in cytosol might be the use of pH-sensitive functions that affect endo-lysosomal membrane stability at low pH. This approach caused faster nuclear Dox accumulation compared to non-pH sensitive liposomes [29-31]. Triggered intracellular Dox release in vitro from CTSL and RGD-TSL accumulated in cytoplasmic vesicles was visualized by live cell confocal microscopy as intracytoplasmic fluorescent nanobursts. The released Dox into lysosomes upon HT resulted in its translocation to the nucleus in the tested tumor cell lines and remained predominantly cytoplasmic in the endothelial cells. Reason for this can be slower lysosomal release of Dox from endothelial cells compared to tumor cells. As a consequence of Dox retention in acidic compartments, we speculate that we could not see improved cytotoxicity with neither CTSL or RGD-TSL when HT was applied. However, when normothermia (NT) was applied, Dox-CTSL were more toxic to all the tested tumor cell lines and endothelial cells than TSL, which again confirms their targeting nature. In accordance with in vitro results, in vivo intravital imaging has shown binding of CTSL and RGD-TSL to tumor vasculature, something which was not observed with TSL. In accordance with Thurston et al. [32] , in both cases binding started approximately 20 min after injection and increased up to 24h. Intravital imaging techniques showed also that although the concentration of both formulations in circulation decreased up to 5h after injection, they were still present in circulation in this time frame that was used for liposomes binding to tumor vasculature. Additionally, the applied HT in vivo could increase liposome extravasation, which was demonstrated by increased fluorescence signal and quantification of images. Besides increased extravasation, HT in vivo triggered massive drug release from both CTSL and RGDTSL (Chapter 3 and 4). The amount of the released drug delivered to the tumor was higher for CTSL compared to TSL, which is due to the targeting nature of this formulation contributing to increased tumor retention and therefore increased drug delivery. The amount of delivered Dox to the tumor from TSL and RGD-TSL was not significantly different. Additionally, both targeted formulations showed stability in vivo in the 5h targeting phase, which was demonstrated by no premature Dox release. Moreover, in the case of Dox-CTSL, a massive vessel destruction could be observed already 24h after liposome injection and was not observed when Dox-TSL were applied, which again proves the specificity of the targeted liposomes for tumor vasculature. This is in

accordance with studies from other groups using non-temperature sensitive [33,34], cationic formulations. The performed pharmacokinetic studies with Dox-CTSL show that despite the targeting nature of liposomes, they were not cleared faster from circulation than TSL (Chapter 6). This is in agreement with the intravital image analysis. Biodistribution studies using initial HT treatment demonstrated that due to the binding features of CTSL, which might be increased upon HT, and HT-induced extravasation, CTSL delivered more Dox to the tumors than TSL. Efficacy studies in B16BL6 melanoma show that HT had a significant effect on CTSL concerning tumor growth inhibition and prolonged survival. However, the efficacy study in B16BL6 did not show a therapeutic effect of the targeted formulation and they did not perform better than non-targeted formulation. Reasons for the lack of suppression in tumor growth as well as prolonged survival could be that the used tumors were too small, the tumor model was fast growing or that we should use a different treatment schedule. Efficacy study in LLC Lewis lung carcinoma with preheat treatment for increased extravasation, demonstrated that two HT treatments show slightly higher tumor inhibition than one HT treatment. A preheating phase for opening up the tumor vasculature for passive accumulation of TSL has already been reported by Li et al [35]. When tumors were preheated for 1h at 41 °C, long-lasting gaps between the tumor vascular endothelial cells were created, thus allowing liposome penetration into the tumor tissue to at least 27.5 μm in radius from tumor vasculature [36]. Therefore, it might be useful to continue performing efficacy study by using two HT treatments in this tumor model including more mice or testing another tumor model with the use of bigger tumors. Therefore, there is still a room for improvement in order to reach an optimal formulation showing optimal therapeutic results.

Hyperthermia treatment scheduling

Hyperthermia is used in these studies as a mean to increase liposome accumulation in the tumor and to trigger drug release. There are two different approaches for triggering drug release: intravascularly or interstitially. Each release approach requires adaptation of the liposomal carrier. Intravascular release approach was suggested by Manzoor et al. [37] and is characterized by HT treatment, which is applied to circulating liposomes thus creating gradient for drug transfer from the high drug concentration in the tumor vasculature towards tumor tissue. This release approach led to a significant increase in penetration depth of doxorubicin into the tumor tissue compared to animals treated with free doxorubicin or Doxil [37]. In this case, fast releasing liposomes that are stable at body temperatures and rapidly release their content at mild temperature, are needed. This treatment schedule also relies on a precise tumor heating. Intravascular release approach has the advantage that here the heterogeneity in tumor vascular permeability and liposome penetration do not play a role [5, 9, 35]. Besides all these, chemotherapeutic drugs with high cellular binding capacity are necessary in order to prevent drug washout. In this approach, there is a continuous drug supply to tumor and endothelial cells as long as heat is applied to the tumor and drug encapsulating liposomes pass the heated area. Additionally, the released drug is easily

available for both tumor and vascular endothelial cells [37-39]. However, the intravascular drug release approach suffers from the possible wash out of the released drug which can cause side effects. Limmer et al. extended the use of intravascular release approach to more hydrophilic drugs, such as gemcitabine [40].

In the current work, we used interstitial release strategy (Chapter 2,3,4 and 5). In this approach, liposomes had to first extravasate through tumor vasculature in order to reach tumor cells. Only after they reach tumor cells, HT trigger is applied for drug release. In this approach, liposome stability and long half-life is even more important. Liposomal size also plays a crucial role for optimal extravasation. Large liposomes have limited penetration depth, preventing drug uptake by tumor cells at a large distance from the vasculature [41, 42]. In order to guarantee a high concentration of delivered drug to the tumor, liposomes must be stable at body temperature. Here, fast drug release and precise tumor heating is not of importance. In this approach, HT can be applied prior to liposome injection in order to increase liposome extravasation and subsequently liposomal drug accumulation. HT is known to increase extravasation is several mouse tumor models and feline sarcoma patients [35, 41, 43, 44]. Since HT can also increase extravasation, the combination between the two approaches might result in even higher concentrations of bioavailable drug. In this case, HT can be used to both trigger drug release intravascularly and increase extravasation, after which the injected liposomes can extravasate through the leaky tumor vasculature. When reaching the tumor cells, second heat treatment can be applied to trigger drug release.

All these treatment schedules seem promising, however, in order to understand which treatment provides best results more studies are needed concerning the possibility of precise heating, the types of used drug and tumor.

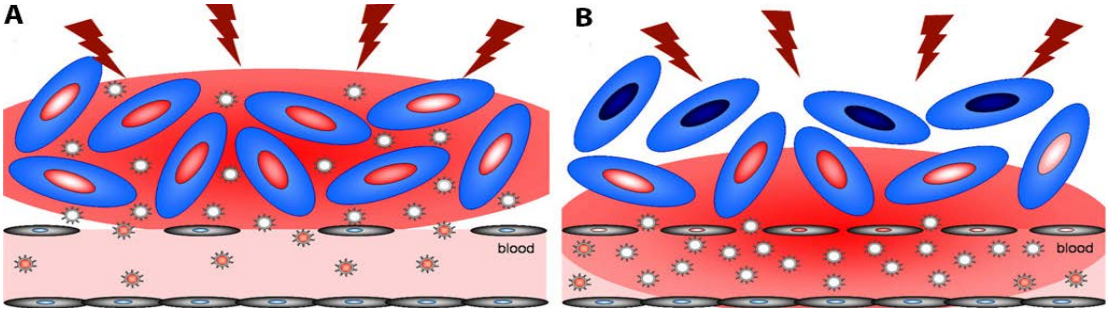

Fig 2. Heating approaches for drug delivery using TSL. A. Interstitial release approach, B. Intravascular release approach. Figures are adapted from Koning et al. [45]

Image-guided drug delivery. Image-guided drug delivery is used in liposomal chemotherapy for monitoring of liposome localization, drug release and/or efficacy of the drug. Therefore, the development of non-invasive imaging techniques monitoring TSL and the release of encapsulated drugs, play a potentially important role in cancer treatment. Nuclear imaging of radiolabelled drugs is one of the most commonly used

ways to image the localization of the drug-encapsulating carrier in tumors [5, 46]. However, this imaging approach cannot be used to image drug release, because the contrast enhancing characteristics of the radioactive agents are the same for the released and the encapsulated drug. Therefore, for this purpose MRI is best used to image both the liposome carrier and the released drug when MRI contrast agent is co-encapsulated with the drug [47-49]. More recently, de Smet et al. have used in their studies the MRI contrast agent Prohance co-encapsulated with doxorubicin in traditional thermosensitive liposomes [50, 51]. They used this approach in HIFU-mediated mild hyperthermia in a HIFU-MRI mediated system. In several studies in a rat, murine and a rabbit tumor models, the combination of TSL with MRI-guided HIFU mediated mild hyperthermia led to increase of doxorubicin concentrations in the tumor when compared to TSL alone and free doxorubicin [50, 52-54]. As the chemotherapeutic efficacy depends on the tumor type and its morphological characteristics, such as vascular permeability and necrosis, image-guided drug delivery might be a powerful tool for personalization of hyperthermia-mediated drug delivery from TSL.

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Summary

In this thesis, temperature-sensitive liposomes and hyperthermia (HT) were used as a tool to increase the efficacy in cancer chemotherapy. Additionally, TSL were targeted for increased specificity to tumors.

Chapter 1 provides a feedback on the study by introducing several topics involved in the thesis. **Chapter 2** is dedicated on the production of novel cationic thermosensitive liposomes (CTSL) encapsulating carboxyfluorescein (CF) as a drug marker. These liposomes proved to be stable at physiological conditions (37 °C) and rapidly released their content when mild HT of 42 °C was applied. They showed higher binding to- and internalization by tumor and endothelial cells than TSL. HT used in vitro was able to cause both extra- and intracellular CF release. In vivo, CTSL bound to tumor and endothelial cells and the applied HT at 43 °C caused a massive CF release.
Chapter 3 focuses on optimization of CTSL containing Dox with regards to drug encapsulation, stability and targeting functions. A CTSL formulation containing 7.5 mol % of the cationic lipid DPTAP proved to be the best for in vitro and in vivo applications. The optimized CTSL showed both targeted and triggered functions. They were able to target both in vitro and in vivo endothelial and tumor cells. In vitro, using confocal microscopy we demonstrated that CTSL are internalized in lysosomes. The performed cytotoxicity assays showed that Dox-CTSL were more toxic to all the tested tumor and endothelial cells than TSL. Additionally, CTSL showed temperature-triggered drug release in vitro and in vivo. In vivo, they were stable at body temperature and abundantly released their drug payload upon heat. Moreover, Dox-CTSL caused massive vessel destruction 24h after injection.
Chapter 4 investigates the development of a novel cRGD-anchoring TSL entrapping Dox. In vitro, these liposomes specifically bound to melanoma and endothelial cells due to their overexpression of αvβ3 integrins. Moreover, the delivered Dox to tumor and endothelial cells was higher for RGD-TSL than TSL. RGD-TSL were internalized into lysosomes. When tumor and endothelial cells were subjected to HT, there was an intracellular burst of the internalized liposomes resulting in drug release into acidic cellular organelles. Some of the released drug could translocate to cellular nuclei. In vivo, RGD-TSL bound to tumor vasculature and the applied HT triggered drug release resulting in drug uptake by tumor and endothelial cells.
Chapter 5 studies the pharmacokinetics, biodistribution and efficacy of Dox-CTSL. The pharmacokinetics shows that targeting of liposomes does not decrease their blood circulation. Dox-CTSL delivered more Dox to the tumor than TSL. Efficacy in B16BL6 murine melanoma shows that HT had a significant effect on CTSL in tumor suppression and prolonged survival. Efficacy in LLC Lewis lung carcinoma tumor model demonstrates that two HT treatments hold promises for a successful treatment option.
Chapter 6 reviews the literature on thermosensitive and targeted liposomes and proposes the recently developed combination of both in one carrier as a promising way of treating cancer.

Chapter 7 discusses the results presented in this thesis in relation to the currently available liposome formulations and treatment options and proposes possible improvements in these targeted and triggered drug delivery nanocarriers.

Samenvatting

In deze thesis werden zogenaamde temperatuur-gevoelige liposomen gebruikt in combinatie met milde hyperthermie voor de behandeling van solide tumoren. Door gebruik te maken van deze liposomen wordt getracht meer van de actieve stof, een chemotherapeuticum, in de tumor te krijgen. Daarnaast werd getracht deze liposomen specifiek naar de tumor te richten.

Hoofdstuk 1 beschrijft de achtergrond van het onderzoek en geeft een inleiding.
Hoofdstuk 2 is toegewijd aan de productie van een nieuw kationisch thermosensitieve liposoom (KTSL). Als model chemotherapeuticum werd carboxyfluorescein (CF) gebruikt. KTSL bleken stabiel onder fysiologische omstandigheden (37°C), terwijl CF zeer snel werd afgegeven indien de liposomen werden blootgesteld aan milde hyperthermie (HT) van 42°C. Deze liposomen bleken tevens beter te binden aan en sneller opgenomen door tumor- en endotheelcellen dan normale TSL. Indien HT werd toegepast gaven de liposomen intra en extracellulair de inhoud af. Ook in vivo werd een verhoogde binding van KTSL aan tumor- en endotheelcellen gezien en veroorzaakte HT een sterke afgifte van CF.
Hoofdstuk 3 beschrijft de optimalisatie van doxorubicine bevattende KTSL met betrekking tot inkapseling, stabiliteit en binding aan de target. Een KTSL formulering waarin 7.5 mol % van het kationische lipid DPTAP bleek het best voor in vitro en in vivo toepassing. Deze liposomen binden in vitro en in vivo gericht aan tumor- en endotheelcellen. We toonde aan met confocale microscopie dat deze liposomen effectief worden opgenomen en belanden in lysosomen. In vitro bleken KTSL met doxorubicine meer actief en beter in staat tumor- en endotheelcellen te doden dan normale TSL. Tijdens bloostelling aan HT geven de liposomen zowel in vitro als in vivo goed doxorubicine af. Tevens bleken deze liposomen in vivo stabiel bij 37°C. Behandeling met KTSL en HT resulteerde een ernstige vaatschade 24 uur na injectie.
Hoofdstuk 4 beschrijft de ontwikkeling van een ander gericht liposoom. Hier wordt circulair RGD (cRGD) gebruikt om TSL met doxorubicine te richten. Deze liposomen binden specifiek aan endotheelcellen maar ook de onderzochte melanoomcellen, aangezien deze cellen αvβ3 integrine tot expressie brengen. Door de aanwezigheid van cRGD op de liposomen konden we meer doxorubicine afleveren in de cellen, waarschijnlijk in de lysosomen. We zagen dat HT resulteerde in snelle afgifte van doxorubicine in organellen in de cel met een relatief lage pH. Van daar uit kon een gedeelte de celkern bereiken. In het proefdier zagen we RGD-TSL gebonden aan de tumorvaatwand en na toepassing van HT een snelle afgifte van doxorubicine wat resulteerde in opname hiervan door zowel tumor- als endotheelcellen.
Hoofdstuk 5 beschrijft de pharmacokinetiek, biodistributie en effectiviteit van KTSL met doxorubicine (DOX-KTSL). Doelgericht maken van het liposoom had geen effect op de circulatietijd, terwijl injectie van DOX-TSL resulteerde in een hoger DOX niveau in de tumor in vergelijking met normale TSL. Studies in muizen met een B16BL6

muizen melanoom laten zien dat HT in combinatie met DOX-KTSL een significant effect heeft op de tumor wat resulteerde in een verlengde overleving. De verkregen resultaten in een LLC Lewis lung carcinoma tumor model laten zien dat vooral het geven van twee HT behandelingen een succesvolle toepassing zou kunnen zijn.

Hoofdstuk 6 geeft een overzicht van de literatuur op het gebied van thermosensitieve en gerichte liposomen. Tevens wordt de recent ontwikkelde combinatie van beide in één liposoom geïntroduceerd als een veelbelovende mogelijkheid voor de behandeling van tumoren.

In Hoofdstuk 7 worden de resultaten gepresenteerd in deze thesis bediscussieerd in relatie tot de liposomale formulering die momenteel beschikbaar zijn en de toepassingen waarvoor ze gebruikt worden. Tevens worden voorstellen gedaan op welke wijze mogelijk verbeteringen kunnen worden aangebracht bij deze gerichte en manipuleerbare nanodeeltjes voor chemotherapie.

by T.L.M. ten Hagen

PhD Portfolio Summary
Summary of PhD training and teaching activities

Name PhD student: Bilyana Dicheva Erasmus MC Department: Surgery Research school: Molecular Medicine	PhD period: 01.08.2009-24.11.2015 Promotor: Prof.A.M.M.Eggermont Supervisor: Dr.G.A.Koning Dr. T.L.M. ten Hagen	
1. PhD training		
	Year	**ECTS**
General academic skills		
- English Biomedical Writing and Communication	2010	2
- Photoshop and Illustrator CS5 Workshop	2012	0.3
- InDesign CS5 Workshop	2013	0.15
- Research management for PhD students	2011	1
- Pubmed Workshop	2010	0.2
- Get out of your lab days	2009	0.6
Research skills		
- Basic Introduction course on SPSS	2011	0.6
- Molecular Medicine	2011	0.7
- Microscopic Image Analysis: From Theory to Practice	2011	0.4
- Image Data Analysis	2011	0.2
In-depth courses		
- In vivo imaging: "From Molecule to Organism''	2008	1.8
- Animal experimentation Article 9	2009	3
- Animal Imaging Workshop by AMIE	2010	1.4
- Biomedical Research Techniques	2010	1.6
- Basic and Translational Oncology	2010	1.8
- Advanced Drug Delivery & Drug Targeting	2011	1.8
- Introduction into Clinical and Fundamental Oncology	2011	2
- Advanced course " Molecular Immunology''	2012	3
- Radiation safety level 5B	2013	3

Presentations		
- Phospholipids in Pharmaceutical Research, Heidelberg, Germany. Poster presentation: Improvement of liposomal drug delivery by enhancement of tumor cell membrane permeability using short- chain sphingolipids	2009	1
- Liposome Workshop/4th Ameland Liposome Workshop. Oral presentation: Thermosensitive cationic liposomes and hyperthermia for drug delivery to endothelial and tumor cells	2009	1
- Imagination 2009, Nijmegen, NL. Oral presentation: Thermosensitive cationic liposomes and hyperthermia for drug delivery to endothelial and tumor cells	2009	1
- Liposome Advances: Progress in Drug and Vaccine Delivery, London, UK. Poster presentation: Cationic thermosensitive liposomes for drug delivery to endothelial and tumor cells	2009	1
- Surgery Staffdag, Rotterdam, NL. Oral presentation: Cationic thermosensitive liposomes as a candidate to improve drug delivery to solid tumors.	2009	1
- 26th Annual Meeting of the European Society of Hyperthermic Oncology, Rotterdam, NL. Oral presentation: Cationic thermosensitive liposomes in combination with hyperthermia for improved drug delivery to endothelial and tumor cells	2010	1
- 2011 Annual Meeting of the Society of Thermal Medicine, New Orleans, Louisiana. Oral presentation: Cationic thermosensitive liposomes - a novel heat-triggered drug delivery approach for endothelial and tumor cells	2011	1
- The 27th Annual Meeting of the Society for Hyperthermic Oncology and the 7th ESHO Educational Day, Aarhus, Denmark. Poster presentation: Cationic thermosensitive liposomes - a novel heat-triggered drug delivery approach for endothelial and tumor cells	2011	1
- Spinoza conference, Amsterdam, NL. Oral presentation: Cationic thermosensitive	2011	1

liposomes for improved drug delivery to tumors. - Micronano conference, Ede, NL. Oral presentation: Cationic thermosensitive liposomes for improved drug delivery to tumors. - Staffdag, Rotterdam, NL. Improving drug delivery to tumors by using cationic thermosensitive liposomes - 11th International Congress of Hyperthermic Oncology (ICHO) & 29th Japanese Congress of Thermal Medicine (JCTM). Poster presentation with a short oral presentation: Novel cationic thermosensitive liposomes for doxorubicin delivery to solid tumors. - 30th Annual STM meeting 2013, Aruba. Oral presentation: Improved doxorubicin delivery to tumors by cationic thermosensitive liposomes. - COST and EMIM meeting 2013, Torino, Italy. Oral presentation: Dual targeted cationic thermosensitive liposomes for improved drug delivery to tumors. Poster presentation: Cationic thermosensitive liposomes to increase doxorubicin delivery to tumors. - ESHO 2013, Munich, Germany. Oral presentation: Cationic thermosensitive liposomes improve drug uptake in solid tumors.	2011 2011 2012 2013 2013 2013	1 1 1 1 1 1
Conferences - Phospholipids in Pharmaceutical Research, Heidelberg, Germany - Liposome Advances: Progress in Drug and Vaccine Delivery, London, UK - 2011 Annual Meeting of the Society of Thermal Medicine, New Orleans, Louisiana - The 27th Annual Meeting of the Society for Hyperthermic Oncology and the 7th ESHO Educational Day, Aarhus, Denmark - 11th International Congress of Hyperthermic Oncology (ICHO) & 29th Japanese Congress of	2009 2009 2011 2011 2012	1 1 1 1 1

Thermal Medicine (JCTM), Kyoto, Japan - 30th Annual STM meeting 2013, Aruba - COST and EMIM meeting 2013, Torino, Italy - ESHO 2013, Munich, Germany - Liposome Workshop/4th Ameland Liposome Workshop - Imagination 2009, Nijmegen, NL - 26th Annual Meeting of the European Society of Hyperthermic Oncology, Rotterdam, NL - Spinoza conference, Amsterdam, NL - Micronano conference, Ede, NL	 2013 2013 2013 2009 2009 2010 2011 2011	 1 1 1 1 1 1 1 1
Seminars and workshops - Lab science day - JNI oncology lecture - Surgery staff dag - MolMed day - Mountain/Sea Liposome Workshop, Ameland, NL - Imagination, Nijmegen, NL - KWF Hyperthermia workshop, Rotterdam, NL - Lab science day - JNI oncology lecture - MolMed day - Surgery staff dag - KWF Hyperthermia workshop, Rotterdam, NL - Lab science day - JNI oncology lecture - Surgery staff dag - MolMed day - KWF Hyperthermia workshop, Rotterdam, NL - Lab science day - JNI oncology lecture - Surgery staff dag - MolMed day - KWF Hyperthermia workshop, Rotterdam, NL - Lab science day - JNI oncology lecture	 2009 2009 2009 2009 2009 2009 2009 2010 2010 2010 2010 2010 2011 2011 2011 2011 2011 2012 2012 2012 2012 2012 2013 2013	 0.2 0.2 0.2 0.2 1 0.2 0.2 0.2 0.2 0.2 0.2 0.2 0.2 0.2 0.2 0.2 0.2 0.2 0.2 0.2 0.2 0.2 0.2 0.2
2.Teaching activities **Supervising practicals and excursions** - Liposome preparation, drug loading optimization and characterization, trainee Mercedeh	 2011	 2

- Targeted liposomes preparation and characterization, trainee Arwin	2012	2
Journal clubs		1

Curriculum Vitae

Education:

1990-1996	SOU “Asen Zlatarov” Montana
1996-2002	Nature Science and Mathematics High School “St. Kliment Ohridski”, Montana ***Main subjects:*** Biology, English and Chemistry
2002-2006	SU “St. Kliment Ohridski”, Biological faculty ***First speciality:*** Biotechnology **Second speciality**: Pedagogy (Biology teacher) ***Degrees obtained:*** Bachelor degree in Biotechnology, Bachelor degree in Pedagogy
10.07.2004- **24.07.2004**	**Summer practice**: morphology, anatomy and physiology of invertebrates and vertebrates
07.07.2005- **20.07.2005**	**Summer practice:** microbiology
20.07.2005- **30.07.2005**	**Summer practice:** industrial biotechnology
2006	University of Perugia, Italy, Faculty of Medicine and Surgery ***Course:*** International Master degree in Biotechnology Medical application */all the subjects are taught in English/*
01.02.2007- **31.07.2007**	**Research internship:** University of Perugia, Faculty of Medicine and Surgery, Department of experimental medicine Supervisor: Professor Maria Pia Viola Magni

	Tutors: Dr. Elissabeta Albi **Research project:** Role of cholesterol on tumour growth
31.01.2008-01.07.2008	**Research internship:** Erasmus MC, Rotterdam, NL, Department of Surgery, Laboratory of Experimental Surgical Oncology Supervisor: Gerben Koning **Research project:** Improvement of liposomal drug delivery by enhancement of tumor cell membrane permeability
01.08.2008-01.08.2009	Lab technician in the Laboratory of Experimental Surgical Oncology, Department of Surgery, Erasmus Medical Center, Rotterdam, The Netherlands **Project:** Biomarkers in pancreatic cancer
01.08.2009-24.11.2015	**Phd:** PhD in the Laboratory of Experimental Surgical Oncology, Department of Surgery, Erasmus Medical Center, Rotterdam, The Netherlands **Project:** Targeted thermosensitive liposomes and hyperthermia for drug delivery to solid tumors

Conferences attended:

24.04-25.04.2008	Imagination 2008, Leiden, NL
08.05.2009	2nd meeting of The Netherlands Platform for Targeted Nanomedicine, Utrecht, NL
11.05-12.05.2009	Phospholipids in Pharmaceutical Research, Heidelberg, Germany. Poster presentation: Improvement of liposomal drug delivery by enhancement of tumor cell membrane permeability using short-

chain sphingolipids

28.09-02.10.2009 Liposome Workshop/4th Ameland Liposome Workshop
Oral presentation: Thermosensitive cationic liposomes and hyperthermia for drug delivery to endothelial and tumor cells

18.11.2009 Imagination 2009, Nijmegen, NL. Oral presentation: Thermosensitive cationic liposomes and hyperthermia for drug delivery to endothelial and tumor cells

12.12-15.12.2009 Liposome Advances: Progress in Drug and Vaccine Delivery, London, UK. Poster presentation: Cationic thermosensitive liposomes for drug delivery to endothelial and tumor cells

09.12.2009 Surgery Staffdag, Rotterdam, NL. Oral presentation: Cationic thermosensitive liposomes as a candidate to improve drug delivery to solid tumors.

20.05-22.05.10 26th Annual Meeting of the European Society of Hyperthermic Oncology, Rotterdam, NL. Oral presentation: Cationic thermosensitive liposomes in combination with hyperthermia for improved drug delivery to endothelial and tumor cells

29.04-02.05.2011 2011 Annual Meeting of the Society of Thermal Medicine, New Orleans, Louisiana. Oral presentation: Cationic thermosensitive liposomes - a novel heat-triggered drug delivery approach for endothelial and tumor cells

25.05-28.05.2011 The 27th Annual Meeting of the Society for Hyperthermic Oncology and the 7th

	ESHO Educational Day, Aarhus, Denmark. Poster presentation: Cationic thermosensitive liposomes - a novel heat-triggered drug delivery approach for endothelial and tumor cells
09.11.2011	Spinoza conference, Amsterdam, NL. Oral presentation: Cationic thermosensitive liposomes for improved drug delivery to tumors.
15.11-16.11.2011	Micronano conference, Ede, NL. Oral presentation: Cationic thermosensitive liposomes for improved drug delivery to tumors.
09.12.2011	Surgery Staffdag, Rotterdam, NL. Improving drug delivery to tumors by using cationic thermosensitive liposomes
28.08-31.08.2012	11th International Congress of Hyperthermic Oncology (ICHO) & 29th Japanese Congress of Thermal Medicine (JCTM).Poster with short oral presentation: Novel cationic thermosensitive liposomes for doxorubicin delivery to solid tumors.
17-21.04.2013	30th Annual STM meeting 2013, Aruba. Oral presentation: Improved doxorubicin delivery to tumors by cationic thermosensitive liposomes.
26-28.05.2013	COST and EMIM meeting 2013, Torino, Italy. Oral presentation: Dual targeted cationic thermosensitive liposomes for improved drug delivery to tumors. Poster presentation: Cationic thermosensitive liposomes to increase doxorubicin delivery to tumors.
19-22.06.2013	ESHO, Munich, Germany. Oral presentation: Cationic thermosensitive liposomes improve drug uptake in solid

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Laboratory skills:	cell culture, TLC, western blotting, gel electrophoresis, PCR, biofermentation and work with bioreactors, basic microbiology procedures, ELISA, confocal and fluorescent microscopy, FACS, animal experiments, immunohistochemistry, liposomes, nanotechnology procedures
Courses:	
27-32.10.2008	In vivo imaging ”From Molecule to Organism”
14.09-02.10.2009	Laboratory Animal Science
03.02-05-02.2010	The 4th Animal Imaging Workshop by AMIE
11.10-15.10.2010	Biomedical Research Techniques
01.11-05.11.2010	Basic and Translational Oncology
01.02-02.02.2011	Molecular Medicine
10.2010-01.2011	Scientific Writing in English for Publication
12.09-14.09.2011	SPSS
22.09/06.10.2011	Research management for phd students
16.11.2011	Microscopic image analysis: From Theory to Practice
21.11-25.11.2011	Advanced drug delivery and Drug targeting
12.12-16.12.2011	Introduction into clinical and fundamental oncology
08.03-16.03.2012	Advanced course “Molecular immunology’’
31.01.2012	Photoshop & Illustrator CS5 Workshop
10.12	Imaging data analysis
06-12.11.12	Advanced course on Radiation safety, level 5B
	InDesign CS5 Workshop
03.2013	
Additional Skills:	
Foreign languages:	English Italian Basic Portuguese Basic Dutch
Computer	Windows, Microsoft Office, Photoshop,

literacy: Illustrator, InDesign, Prism, Internet

Awards:

1. ESHO-Magforce student award for biology, Rotterdam, NL, 2010.
2. Young Investigator Award at the 11th International Congress of Hyperthermic Oncology & the 29th Japanese Congress of Thermal Medicine, Kyoto, Japan, 2012.
3. ESHO-Rosner Award Winner for biology, 2012.
4. New Investigator Travel Award at STM (Society for Thermal Medicine)in Aruba, 2013.

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Publication List

1. **Dicheva BM**, ten Hagen TL, Li L, Schipper D, Seynhaeve AL, van Rhoon GC, et al. Cationic thermosensitive liposomes: a novel dual targeted heat-triggered drug delivery approach for endothelial and tumor cells. Nano Lett 2013 Jun 12;13(6):2324-31.

2. **Dicheva BM**, Koning GA. Targeted thermosensitive liposomes: an attractive novel approach for increased drug delivery to solid tumors. Expert Opin Drug Deliv 2014 Jan;11(1):83-100.

3. **Dicheva BM**, ten Hagen TL, Schipper D, Seynhaeve AL, van Rhoon GC, Eggermont AM, et al. Targeted and heat-triggered doxorubicin delivery to tumors by dual targeted cationic thermosensitive liposomes. J Control Release 2014 Dec 10;195:37-48.

4. **Dicheva BM**, Ten Hagen TL, Seynhaeve AL, Amin M, Eggermont AM, Koning GA. Enhanced Specificity and Drug Delivery in Tumors by cRGD - Anchoring Thermosensitive Liposomes. Pharm Res 2015 Jul 23.

5. **Dicheva BM**, Seynhaeve AL, Soulie T, Eggermont AMM, ten Hagen T, Koning GA. Pharmacokinetics, tissue distribution and therapeutic effect of cationic thermosensitive liposomal doxorubicin upon mild hyperthermia. Accepted in Pharm Res.

6. Seynhaeve AL, **Dicheva BM**, Hoving S, Koning GA, ten Hagen TL. Intact Doxil is taken up intracellularly and released doxorubicin sequesters in the lysosome: evaluated by in vitro/in vivo live cell imaging. J Control Release 2013 Nov 28;172(1):330-40.

7. van der Zee JA, ten Hagen TL, Hop WC, van Dekken H, **Dicheva BM**, Seynhaeve AL, et al. Differential expression and prognostic value of HMGA1 in pancreatic head and periampullary cancer. Eur J Cancer 2010 Dec;46(18):3393-9.

8. van der Zee JA, Ten Hagen TL, Hop WC, van Dekken H, **Dicheva BM**, Seynhaeve AL, et al. Bcl-2 associated anthanogen-1 (Bag-1) expression and prognostic value in pancreatic head and periampullary cancer. Eur J Cancer 2013 Jan;49(2):323-8.

9. van der Zee JA, van Eijck CH, Hop WC, Biermann K, **Dicheva BM**, Seynhaeve AL, et al. Tumour basement membrane laminin expression predicts outcome following curative resection of pancreatic head cancer. Br J Cancer 2012 Sep 25;107(7):1153-8.

10. van der Zee JA, van Eijck CH, Hop WC, van Dekken H, **Dicheva BM**, Seynhaeve AL, et al. Angiogenesis: a prognostic determinant in pancreatic cancer? Eur J Cancer 2011 Nov;47(17):2576-84.

11. van der Zee JA, van Eijck CH, Hop WC, van Dekken H, **Dicheva BM**, Seynhaeve AL, et al. Expression and prognostic significance of thymidylate synthase (TS) in pancreatic head and periampullary cancer. Eur J Surg Oncol 2012

Nov;38(11):1058-64.
12. van Lummel M, van Blitterswijk WJ, Vink SR, Veldman RJ, van der Valk MA, Schipper D, **Dicheva BM**, Eggermont AM, ten Hagen TL, Verheij M, Koning GA. Enriching lipid nanovesicles with short-chain glucosylceramide improves doxorubicin delivery and efficacy in solid tumors. FASEB J 2011 Jan;25(1):280-9.