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# Triggered doxorubicin release in solid tumors from thermosensitive liposome-peptide hybrids: Critical parameters and therapeutic efficacy

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**Temperature-sensitive vesicles designed by inclusion of leucine zipper peptides within a lipid bilayer (Lp-Peptide hybrids) encapsulating Doxorubicin (DOX) have been reported. Intravenous administration of these constructs prolonged blood circulation kinetics and increased tumor accumulation *in vivo* with local mild hyperthermia. In this study, the biological activity of the DOX-loaded Lp-Peptide hybrid vesicles was further investigated at the cellular level and *in vivo* compared to lysolipid-containing temperature-sensitive liposomes (LTSL) and traditional temperature-sensitive liposomes. Lp-Peptide vesicles were not toxic to cell cultures at 37°C, while effective cancer cell toxicity was observed after 1 hr of heating at 42°C. The activity of Lp-Peptide vesicles *in vivo* was studied using two different heating protocols to obtain tumor intravascular or interstitial drug release. Lp-Peptide vesicle treatment allowing intravascular DOX release showed equally effective tumor growth retardation and survival to that of LTSL treatment. The Lp-Peptide vesicles also offered therapeutic responses using the alternative heating protocol to maximise drug release within the tumor interstitium. Matching the drug release kinetics of temperature-sensitive vesicles with the heating protocol applied is considered the most critical factor to determine therapeutic efficacy in the clinical translation of such modalities.**

The design of drug delivery technologies with controllable drug retention and drug release properties is a challenge. Despite a decrease in the risk of drug-associated toxicity[1] and delivering more drug to the tumor tissue by encapsulation inside long-circulating liposomes,[2] the therapeutic efficacy of liposomal Doxorubicin (DOX) is not dramatically enhanced because of limited drug release from liposomes.[3–5] Local hyperthermia[6] represents one external trigger that can improve liposomal drug release by increasing their extravasation into the tumor,[7] triggering content release[8] and potentiating a chemotherapeutic effect.[9]

**Key words:** hyperthermia, vesicles, chemotherapy, cancer therapy, temperature-sensitive, nanoparticles
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Different temperature-sensitive liposomes (TSL) have been developed following the original work by Yatvin and Weinstein.[10,11] This "traditional" TSL system has since been further developed for increased blood circulation half-life[12,13] and serum stability.[14,15] In addition drug release kinetics at the lipid bilayer phase transition temperature have been enhanced by inclusion of additional lipid components, such as lysolipids or oligoglycerol.[16–18]

The ultrafast drug release capabilities of some TSL offer a new paradigm in liposomal DOX release compared to nontemperature-sensitive liposomes (NTSL), such as the clinically used Doxil®. Previous studies suggested that ultrafast drug release within the tumor vasculature should be achieved upon reaching the heated tumor tissue if TSL were injected just prior to or during HT.[19] This type of drug release is termed "intravascular" release approach and its main advantage is that DOX-loaded liposomes do not need to extravasate from the vasculature and accumulate within the tumor interstitium.[8,20] The most clinically successful intravascular triggered release system is the lysolipid-containing TSL (LTSL) developed by Needham *et al.*[17,19] The ultrafast DOX release triggered from LTSL within the tumor vasculature after exposure to mild HT (80% release within a few seconds at 42 °C)[21,22] increased the exposure of tumor endothelial cells to DOX causing destruction of tumor vasculature and improvements in therapeutic efficacy.[6,8,17,23,24] This system is currently under evaluation in late-stage clinical trials under trade name ThermoDox®.[25]

**What's new?**

**An enticing drug delivery approach has been to enclose a biologically-active small molecule within temperature-sensitive vesicles that can travel to the tumor site and release their contents on-demand, when heated. A new type of such thermosensitive vesicle type, based on the hybrid membrane formation between lipids and peptides (Lp-Peptide hybrids) carrying doxorubicin is studied here, along with the critical conditions that could elicit the most efficient tumor-killing in mice bearing human tumors. The new Lp-Peptide vesicles showed that were able to halt tumor growth more effectively when triggered to release the drug intravascularly compared to interstitially released drug. The key point demonstrated is that Lp-Peptide hybrid vesicles have the ability to release doxorubicin using different heating and administration protocols that can maximize treatment options without compromising effectiveness.**

The combination of TSL and HT may also be tailored to achieve drug release within the tumor interstitium after adequate extravasation, assuming that sufficient drug stability and retention in the blood stream is achieved.[7,26] HT has also been reported to offer a synergistic effect in enhancing liposome extravasation[7,26,27]; therefore application of an initial HT prior to TSL administration could increase tumor vascular permeability and interstitial accumulation. Once the liposomes extravasate into the tumor interstitium, a second HT can be used to trigger drug release from these liposomes.[28] This type of heating protocols constitutes what is termed "interstitial" release approach.

Recently, we described the development of a previously unreported lipid-peptide hybrid vesicle system (Lp-Peptide) that was designed to behave in a similar way to LTSL by enhancing DOX release on mild HT, while maintaining stability and drug retention in the blood.[29] Lp-Peptide vesicles were engineered by anchoring temperature-sensitive leucine zipper peptide molecules within the liposomal lipid bilayer. The self-assembly of the peptide into super-helix coiled-coil structure at low temperatures and its dissociation by mild HT were thought to be responsible for triggering drug release. Our previous findings showed that DOX tumor accumulation from Lp-Peptide hybrids immediately after HT was equivalent to that obtained from LTSL, in addition to a significant (threefold) increase in DOX tumor concentration 24 hr after HT compared to LTSL. Taking these together, we hypothesised that the combination of rapid DOX release and substantial tumor accumulation of DOX constituted a good candidate system to interrogate further intravascular and interstitial drug release approaches.

In this study, the biological and therapeutic activity of DOX-encapsulating Lp-Peptide vesicles were studied by intravascular or interstitial triggered drug release protocols in combination with HT. To determine the suitability between HT protocols with the drug release capabilities of TSL, different types of TSL were included for comparison. Traditional TSL (TTSL) characterised by extended blood circulation half-life and intermediate drug release kinetics[14,30] and lysolipid-containing TSL (LTSL) that offer a rapid drug release profile[22] with short blood circulation time[19,30] were included. We investigated the comparative tumor accumulation of DOX using live optical imaging, biological activity and therapeutic efficacy of all three TSL systems using a human colorectal adenocarcinoma (SW480) xenograft model.

## Material and Methods

### Materials

Leucine zipper peptide was purchased from Peptide Synthetics (Peptide Protein Research, Hampshire, UK). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-disearolyl-*sn*-glycero-3-phosphocholine (DSPC), 1-stearoyl-*sn*-glycero-3-phosphocholine (MSPC), hydrogenated soy phosphatidylcholine (HSPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000 (DSPE-PEG2000) were kind gifts from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol, Chloroform, DOX hydrochloride and Sepharose CL-4B were obtained from Sigma (UK). 1,1′-Dioctadecyl-3,3,3′,3′-Tetramethylindocarbocyanine Perchlorate (DiI) was purchased from Invitrogen Detection Technologies. Polycarbonate extrusion filters (Whatman) 800, 200, 100 and 80 nm were form VWR, UK.

### Preparation of TSL

Preparation of the three different types of TSL is explained in details in the Supporting Information.

### Lp-peptide hybrids interaction with B16F10 and HUVEC cells

Details of cellular biocompatibility of Lp-Peptide hybrids on B16F10 cells (murine melanoma) and HUVEC cells (primary human umbilical vein endothelial cells) are explained in the supporting information.

### Cellular uptake studies

The cellular uptake of DiI-labelled (5% mol) Lp and Lp-Peptide hybrids was studied in B16F10 cells at 20,000 cells per well on glass cover-slip for 3 and 24 hr at 37 °C. At the end of incubation cells were washed with PBS and fixed with PFA 4% (Thermoscientific, UK) for 10–15 min at room temperature then mounted using 3 μl Vectashield mounting medium with DAPI H-1200 (Vector Laboratories). Imaging of cellular uptake was done using confocal laser scanning microscopy (CLSM) Zeiss LSM 710 (Obserkochen, Germany) using EC Plan-Apochromat 40×/1.3 oil. DAPI staining of nucleus was detected at 405 nm laser excitation source and

410 nm output filter, whereas DiI and Cy3 were imaged at 514 nm laser excitation source and 585 nm output filter.

### Cytotoxicity of DOX-loaded TSL

*In vitro* cytotoxicity of DOX-loaded TTSL, LTSL, Lp-Peptide 200:1 hybrids was compared with B16F10 and SW480 (6,000 and 10,000 cells per well, respectively) using MTT reduction assay. To evaluate the stability and the thermal sensitivity of Lp-Peptide hybrids compared with TTSL and LTSL, liposomes were heated for 1 hr at 42 °C in complete media before incubation with the cells and compared with non-heated liposomes.

Cells were then treated with both heated and nonheated liposomes at either 1 and 10 μM DOX concentrations (0.0145 and 0.145 mM lipid) for 3 hrs at 37 °C in $CO_2$ Incubator. These concentrations were selected to cover the expected range of both DOX and lipids concentration after *in vivo* administration. At the end of treatment, cells were washed and replaced with liposomes-free media and incubated at 37 °C for either 24 or 48 hr. Cell viability was then assessed with MTT assay as explained earlier and the results were expressed as percentage of untreated control cells.

### Optical imaging of DOX accumulation into SW480 tumor

*In vivo* optical imaging was used to study the accumulation of DOX into SW480 tumor-bearing mice by looking at the optical fluorescence signal of DOX in live animals. TTSL, LTSL and Lp-Peptide hybrids injected mice using both intravascular and interstitial release protocols were imaged using IVIS Lumina II imaging system (Caliper Life Sciences Corp., Alameda, CA). For intravascular release protocol IVIS acquisition was performed 1 and 24 hr after injection, while for the interstitial release protocol images were taken 24 hr after injection, before and after second HT. IVIS acquisition was not performed at 1 hr time point for the interstitial protocol as no triggered drug release took place 1 hr after injection with this protocol. Images were taken at 500 nm/DsRed excitation and emission filters and corrected by subtraction from background images (taken from control, un-injected mice) at 430 nm excitation wavelength and GFP emission filter to exclude the contribution of any tissue autoflorescent. DOX fluorescent intensity at the tumor site was quantified by drawing a region of interest (ROI) that covers the tumor-bearing leg and expressed as total efficiency. However, it has to be emphasised that great caution should be taken in the analysis of such "semi-quantitiative" estimations of fluorescence intensity that are not able to offer absolute and accurate quantification of DOX concentrations in the tumor, blood or other tissues as alternative techniques can (e.g., scintillation counting, HPLC).

### Tumor growth retardation and survival studies

The therapeutic activity of Lp-Peptide hybrids was studied using two HT protocols to mimic intravascular and interstitial drug release protocols (Scheme 1) in comparison with TTSL and LTSL. SW480 tumor bearing mice were treated with single dose of TTSL, LTSL and Lp-Peptide 200:1 hybrids (5 mg/kg DOX) and control mice in both protocols are mice treated with HT only.

For the intravascular drug release, only a single HT session (1 hr) was applied immediately after TSL intravenous injection to trigger drug release from TSL within the heated tumor vasculature. Alternatively, for the interstitial release protocol, two HT sessions were applied. The first HT (1 hr) was applied before the injection of TSL to increase the local tumor endothelial cell permeability; 24 hr after TSL injection a second HT session (30 min) was applied to trigger drug release interstitially from TSL accumulated within the tumor. Mice were also examined for any change in body weight or signs of toxicity twice a week. Therapy experiments were terminated when tumors volume reached 1000 $mm^3$.

### Histopathological analysis

To assess for any histological changes as a result of treatment with DOX-loaded TSL, major organs were collected from treated mice and compared with control mice. Mice were sacrificed by cervical dislocation 3–5 weeks after injection with the exception of mice treated with TTSL liposomes (interstitial release protocol) who had to be euthanized earlier (10 days after injection) because of the severe weight loss (15–20% of initial weight). Tissue samples were fixed in neutral buffered formalin and processed routinely into paraffin before sectioning and staining with Hematoxylin and Eosin (H & E).

### Statistical analysis

Statistical analysis of the data was performed using Graph Pad Prism software. Two-tailed unpaired Student's *t* test and one-way analysis of variance followed by the Tukey multiple comparison test were used and $p$ values $<0.05$ was considered significant. Statistical analysis of the therapy data was performed and evaluated by both parametric (ANOVA) and nonparametric (Wilcoxon/Kruskal-Wallis) tests to indicate no differences in the resulting conclusions.

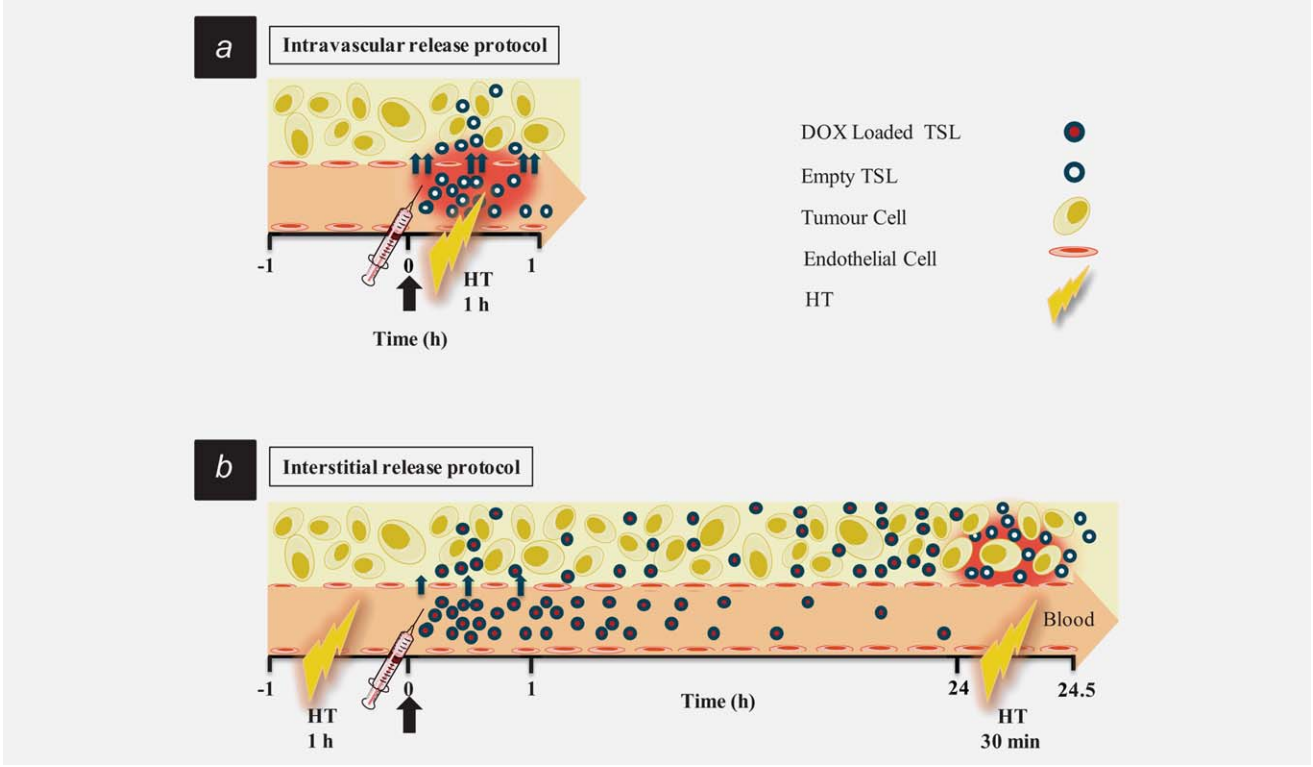
## Results

### Preparation of TSL

Lp-Peptide hybrids were prepared as previously described by incorporation of the peptide into DPPC:DSPC:DSPE-$PEG_{2000}$ (90:10:5) mol/mol at 200:1 lipid:peptide molar ratio and compared to LTSL and TTSL liposomes. Figure 1 shows that all three types of TSL had a hydrodynamic diameter of around 100 nm with low mean size distribution (polydispersity index) and were moderately negatively charged.

### Interaction of Lp-peptide hybrids with cells

The biocompatibility of liposomes at therapeutic doses makes them attractive vehicles for drug delivery. To make sure the presence of leucine zipper peptide in Lp-Peptide hybrid system did not compromise the biocompatibility of tumor and endothelial cells, we tested their effect *in vitro* on the viability of B16F10 cells and HUVEC cells, respectively. Supporting




**Scheme 1. Schematic presentation the different heating protocols applied to compare the tumor DOX accumulation and the therapeutic activity of TSL.** The combination of hyperthermia and liposome systems can be used to enhance the drug release from TSL in two different protocols based on the timing between liposomes administration and heat application. (*a*) In the intravascular release protocol, TSL are administered during the heating process, resulting in drug release inside blood vessels when reaching the heated area (drug release is presented by red gradient seen in the blood vessels). This process is then followed by drug taken up by both tumor and endothelial cells. (*b*) The increased vascular permeability of the blood vessels in response to the first HT treatment increases the level of liposomes accumulation in the tumor. The interstitial release approach takes advantage of the fact that stealth small size liposomes have the ability to extravasate the malformed tumor vasculature compared to normal blood vessels. After tumor accumulation, a second heating is applied to trigger drug release interstitially (drug release is represented by the red gradient close to tumor cells). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]



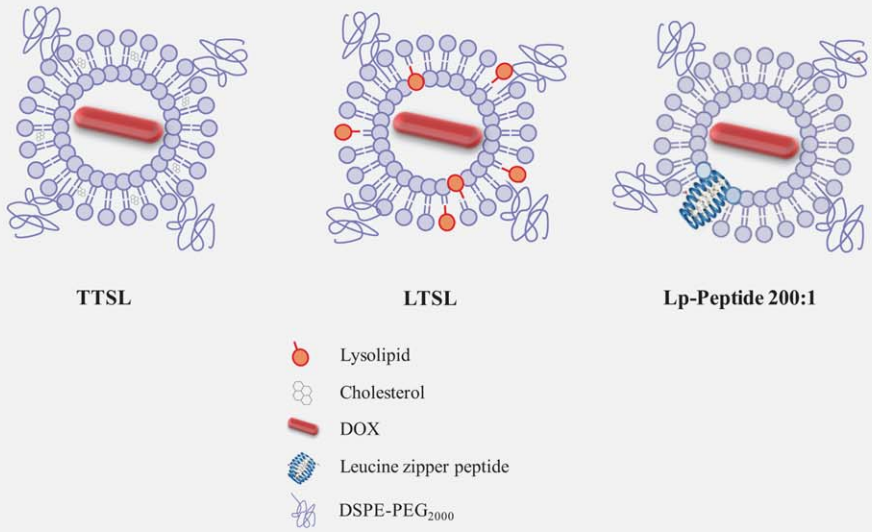
Information Figures S1A and S1B shows that the incubation of both Lp and Lp-Peptide hybrids with B16F10 cells up to 24 hr did not significantly affect their viability at all the concentrations tested. Similar to B16F10 cells, both Lp and Lp-Peptide hybrids (0.145 mM) did not show any toxic effect on HUVEC cells after 4 hr incubation (Supporting Information Fig. S1C) and only moderate reduction in cell viability by 10% was observed after 24 hr incubation. In both cases, no significant differences were seen between liposomes with and without peptide, which confirmed the biocompatibility of the hybrids.

Cellular uptake of DiI-labeled Lp-Peptide hybrids was then studied with CLSM after 3 and 24 hrs incubation with B16F10 cells. Figure 2 shows that both Lp and Lp-Peptide hybrids were taken up by the cells to the same extent. Cellular uptake was both concentration and time dependent and the highest uptake was observed after 24 hr incubation at 0.5 mM lipid concentration. Overall the Lp-Peptide hybrids did not show any cytotoxic side-effect on the cells.

### Cytotoxicity of DOX-loaded TSL

The next step was to evaluate the cytotoxic activity of DOX-loaded hybrids compared with other TSL and their potential for triggered drug release. Cytotoxicity was assessed by measuring the cellular viability with MTT assay in B16F10 and SW480 cell lines. The choice of these cell lines was based on their sensitivity to DOX treatment and their cell division rate to have good correlation with the *in vivo* therapeutic data (Supporting Information Fig. S2). Preheating the TSL before cell treatment was used instead of heating after cell treatment. This will allow the cells to expose to the free DOX and TSL-released DOX for the same time. In this way, we can avoid variability in the percentage of cell viability for the same concentration of free DOX and TSL-released DOX (Supporting Information Fig. S3). Figure 3*a* shows that incubation with TSL for 3 hr at 10 μM DOX concentration without preheating did not significantly affect cell viability of both B16F10 cells and SW480 in agreement with previous studies.[31] Exception from that were LTSL liposomes, which

a




b

| Liposomes | Composition mol/mol % | Hydrodynamic Diameter (nm) | Polydispersity Index (PDI) | Zeta Potential (mV) |
|---|---|---|---|---|
| TTSL | DPPC:HSPC:Chol:DSPE -PEG $_{2000}$ (54:27:16:3) | 121.00 ± 1.67 | 0.14 ± 0.01 | -22.87 ± 0.47 |
| LTSL | DPPC:MSPC:DSPE -PEG $_{2000}$ (90:10:4) | 112.00 ± 2.78 | 0.15 ± 0.01 | -21.70 ± 0.55 |
| Lp-Peptide 200:1 | DPPC:DSPC: DSPE -PEG $_{2000}$: Peptide (90:10:5:0.5) | 128.27 ± 2.70 | 0.27 ± 0.03 | -22.07 ± 0.75 |

The results are expressed as mean ± , n=3.
DPPC : 1,2-dipalmitoyl-sn-glycero-3-phosphocholine
DSPC : 1,2- distearoyll-sn-glycero-3-phosphocholine
MSPC : 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine(lysolipid).
HSPC : Hydrogentated Soy phosphatidylcholine
DSPE -PEG $_{2000}$: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000.
CHOL : Cholesterol

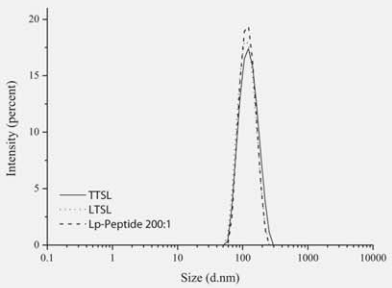



**Figure 1. Design and Characterization of TSL.** (*a*) Schematic presentation of different types of TSL used in this study; TTSL, LTSL and Lp-Peptide 200:1. (*b*) Size, PDI and surface properties of TSL. Data represented as mean ± STD ($n = 3$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

resulted in significant cytotoxicity without HT on B16F10 cells after 24 hr incubation at this concentration tested as a result of their leaky character.[32] However, the 10 μM DOX concentration tested here for *in vitro* cytotoxicity assays is much higher than what would be expected from spontaneous drug leakage without hyperthermia *in vivo* because of the rapid clearance of free DOX.

After a prolonged incubation time (48 hr), reduction in cell viability observed from LTSL and other TSL (Fig. 3*b*) was thought to be due to intracellular drug release. Alternatively, incubating the cells with preheated TSL of all types resulted in significant enhancement in cellular toxicity (almost identical to the effect observed for free DOX) indicating complete drug release from liposomes (Fig. 3).

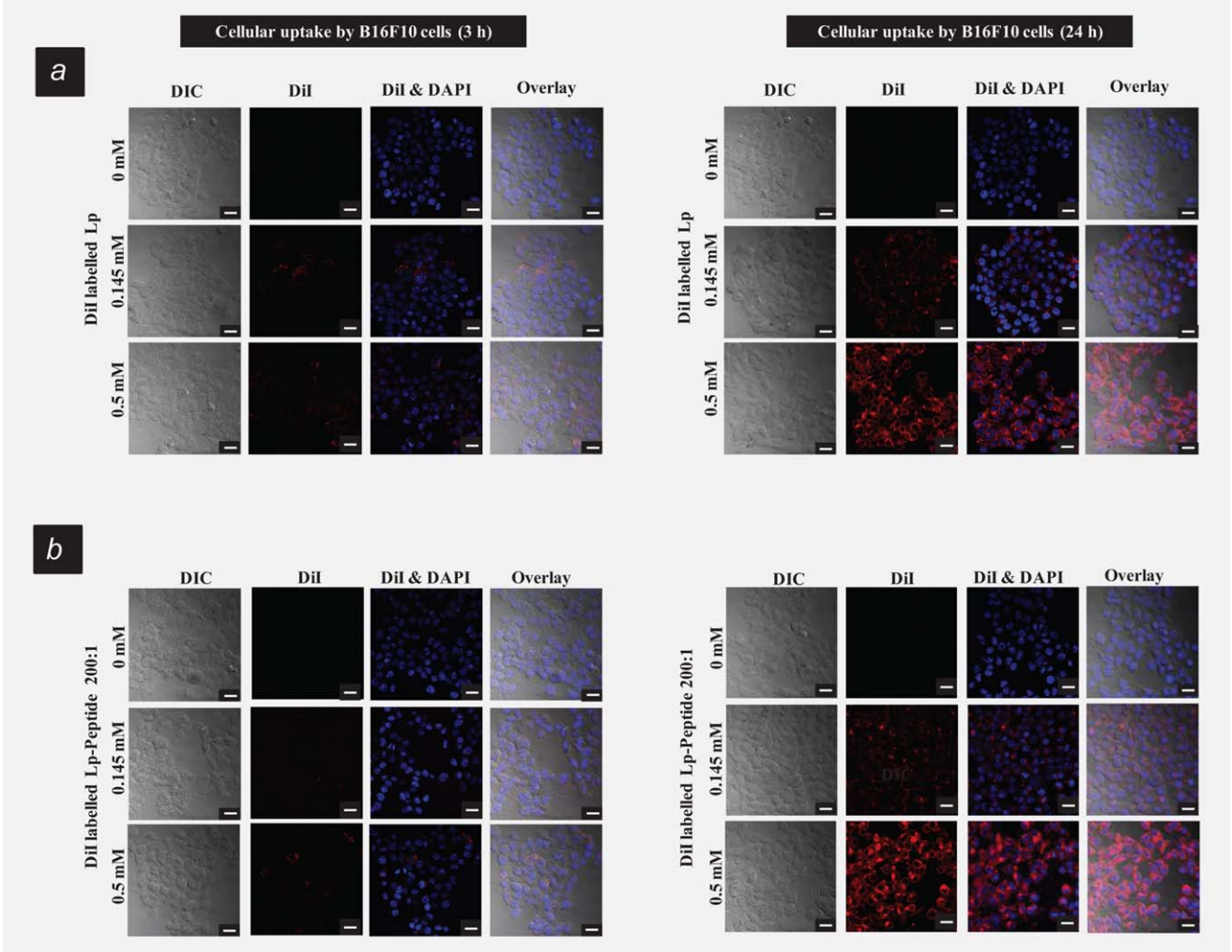



**Figure 2. Cellular uptake studies of Lp and Lp-Peptide 200:1 into B16F10 cells.** Confocal microscopy imaging of monolayer of B16F10 cells showed the uptake of DiI-labelled; **(*a*)** Lp and **(*b*)** Lp-Peptide hybrids 200:1 after 3 and 24 hr incubation at 37 °C. Red signal represents the uptake of liposomes (signals from DiI-labeled liposomes). Colocalization with DAPI staining (blue) of the nucleus is shown in the overlay images. Scale bar is 20 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

In comparison, no significant cytotoxicity was observed with NTSL (included as a negative control) both with and without preheating because of the slow drug release even under mild HT. The same trend was observed in both cell lines studied with slight variation due to the difference in the sensitivity among cell lines to DOX. Similar data were observed with B16F10 cells treated at 1 μM DOX concentration, however, no clear cytotoxic activity on SW480 cells was observed with both heated and nonheated TSL at this concentration (Supporting Information Fig. S4).

### Optical imaging of DOX accumulation into SW480 tumor

*In vivo* optical imaging was performed to compare the accumulation of DOX into SW480 tumor-bearing mice treated with these three types of TSL applying both, intravascular and interstitial drug release protocols. IVIS imaging of animals treated with the intravascular release protocol showed that the highest tumor DOX accumulation was achieved from LTSL and Lp-Peptide hybrids due to their fast drug release properties (Fig. 4*a*-i). Quantification of DOX fluorescent signal at the tumor site showed that Lp-Peptide hybrids resulted in equivalent DOX accumulation to LTSL and significantly higher than TTSL liposomes (Fig. 4*b*-i). 24 hr after injection, reduction in DOX signal at the tumor site was observed from all TSL indicating a degree of wash-out of DOX molecules from the tumor.[33]

With the interstitial drug release protocol, IVIS imaging was performed 24 hr after injection and before and after the second HT. Unlike with intravascular drug release LTSL liposomes resulted in significantly low DOX accumulation compared to Lp-Peptide hybrids and TTSL (Figs. 4a-ii and *b*-ii). This can be understood based on the differences in blood circulation profile and the ability to retain DOX after *in vivo* administration.[32,34] In addition, the application of second HT did not significantly affect the overall DOX accumulation levels.

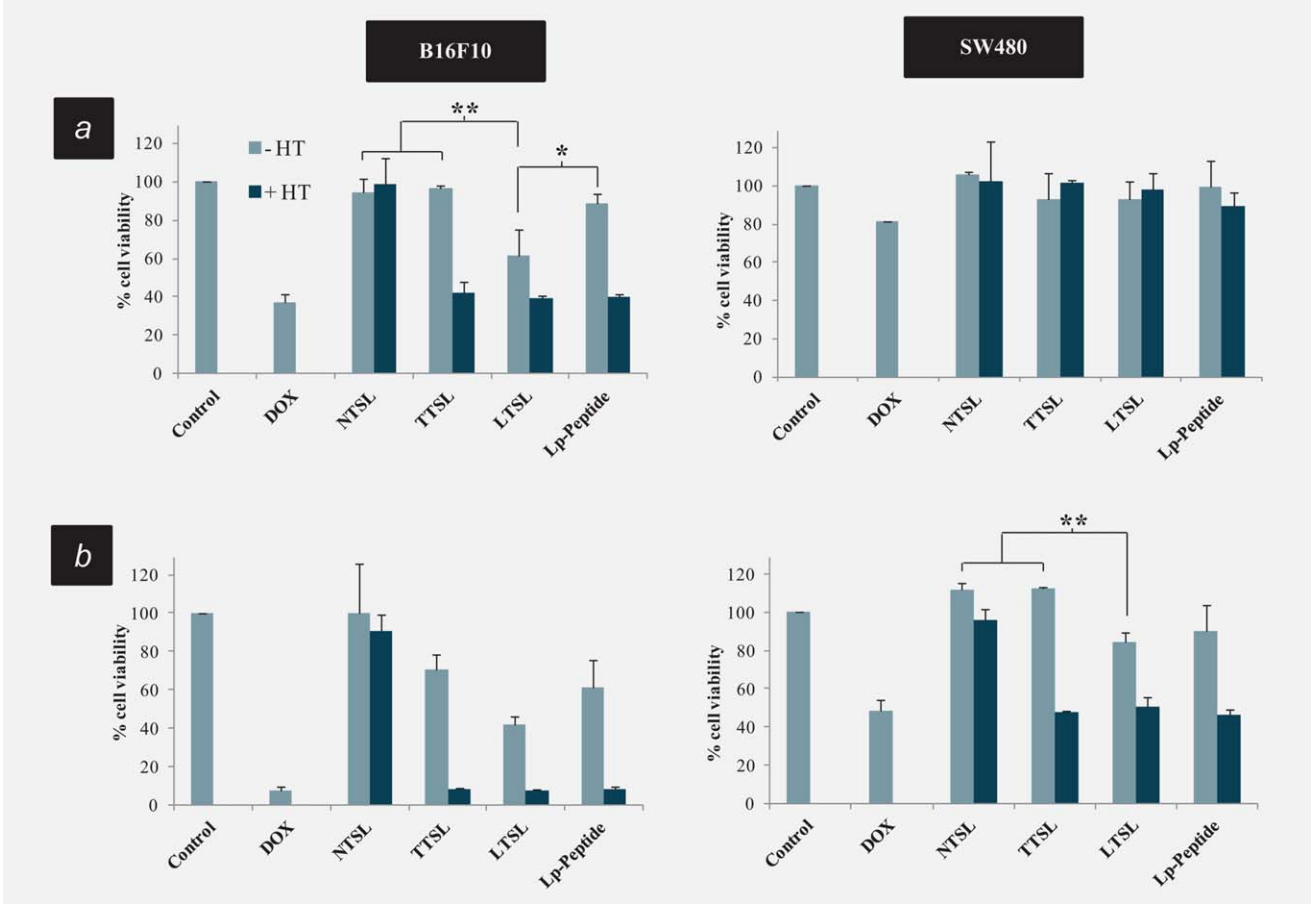



**Figure 3. Cytotoxic activity (MTT assay) of different types of TSL in comparison to NTSL.** The cytotoxic activity of DOX loaded TSL was studied at 10 μM DOX concentration (0.145 mM lipid) on B16F10 and SW480 cells after: **(*a*)** 24 hr and **(*b*)** 48 hr incubation. To study the effect of HT on drug release and cytotoxicity of TSL, liposomes where heated for 1 hr at 42 °C prior to cell treatment and compared with non-heated liposomes. Cell monolayers were treated for 3 hr then the liposome-containing media were removed and replaced with fresh media. MTT assay was performed at 24 and 48 hr after treatment and expressed as percentage of cell viability. Results represented as average ± STD of at least two independent experiments (6 wells per treated group). * indicates $p < 0.05$ and ** indicates $p < 0.01$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

### Tumor growth retardation and survival studies (intravascular vs.interstitial drug release protocol)

Based on the change in tumor volume (Fig. 5*a*), we observed that all animals treated for intravascular release showed significant tumor growth retardation compared to control mice. Lp-Peptide hybrids and LTSL liposomes had equivalent therapeutic activity and were significantly more effective compared to TTSL because of their ultrafast release properties (In agreement with the IVIS tumor accumulation data). No signs of toxicity were observed from all treated groups using the intravascular release protocol and no significant change in the weight of the mice was observed (Fig. 5*b*).

When the HT protocol was changed to release DOX interstitially, we observed that TTSL treatment showed tumor growth control up to 10 days after treatment because of their long blood circulation profile that resulted in the highest DOX tumor accumulation as can be seen from IVIS. However, this was also accompanied with significant weight loss that suggested nonspecific systemic toxicity (Fig. 5). LTSL treatment, however, did not show any improvement compared with control. This effect is mainly because of the limited serum stability and short blood circulation that limits LTSL tumor accumulation.[34] The best tumor growth retardation obtained from the interstitial protocol was from Lp-Peptide hybrids. Lp-Peptide was more effective than LTSL because of their longer blood circulation that resulted in good tumor accumulation (Fig. 5*a*). In comparison to TTSL, no signs of toxicity or change in body weight were observed with LTSL and Lp-Peptide treated mice (Fig. 5*b*).

Similar findings were observed in survival rates that agreed with the tumor growth delay and DOX accumulation data (Fig. 5*c*). With the case of intravascular release, significant increase in life span ($p < 0.001$) was achieved from LTSL and Lp-Peptide hybrid treatment (Supporting Information Table S1). However, with interstitial drug release, TTSL treated mice exhibited 50% reduction in survival compared to the control group and significant weight loss (>15% or more drop in body weight), that indicated systemic toxicity

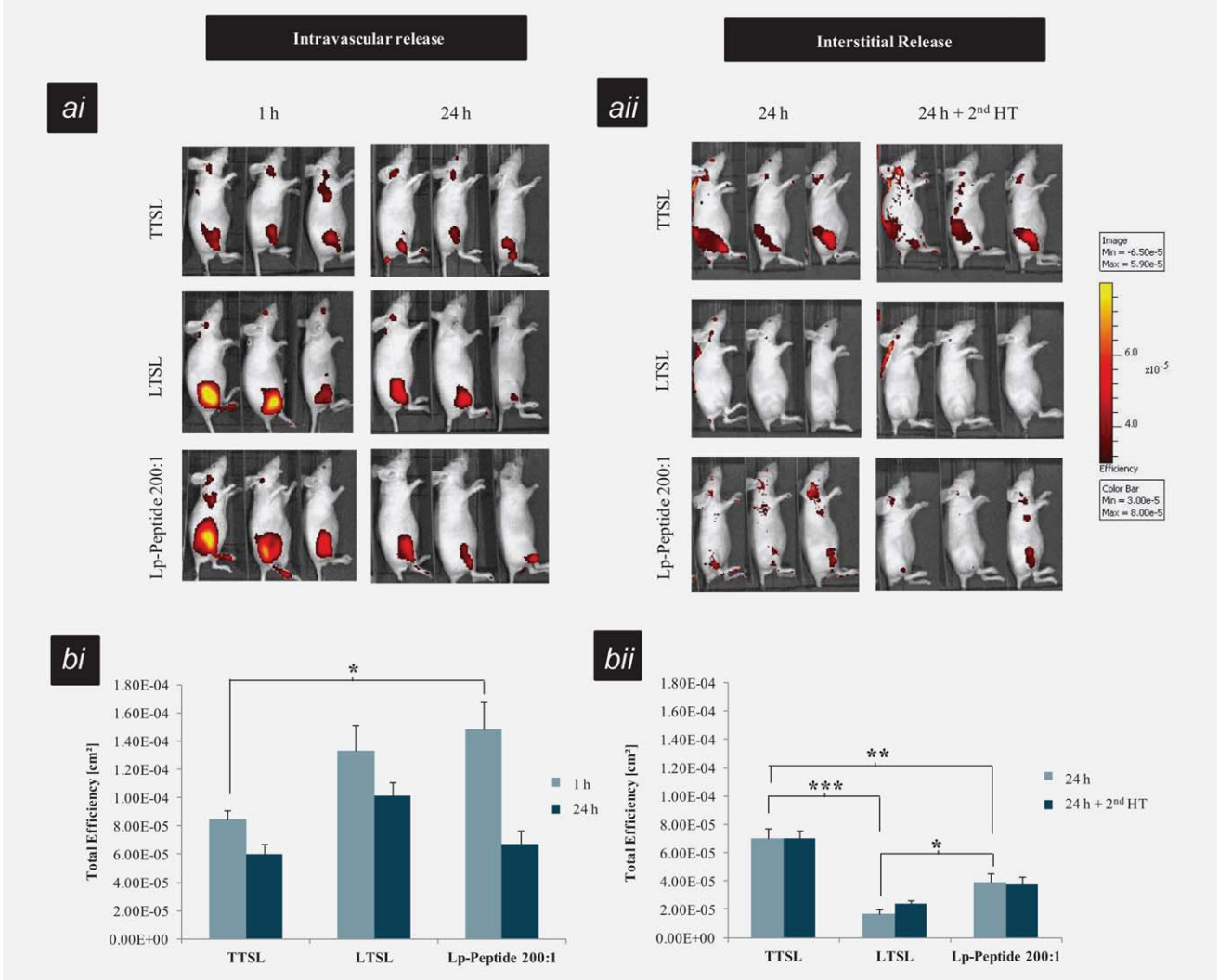



**Figure 4.** *In vivo* optical imaging of DOX fluorescence in athymic mice treated with the intravascular and interstitial release protocol. For the intravascular release protocol TTSL, LTSL and Lp-Peptide 200:1 were injected into SW480 tumor-bearing mice followed by immediate HT at 42 °C to trigger intravascular drug release. Whereas, for the interstitial release protocol SW480 tumor-bearing mice were exposed to 1 hr local HT (42 °C) prior to injection with TTSL, LTSL and Lp-Peptide 200:1 liposomes. 24 hr after injection second session of local HT was applied to trigger interstitial drug release after tumor accumulation. Mice were then imaged with IVIS Lumina II imaging system 1 and 24 hr after injection and heating for the intravascular protocol and 24 hr after injection before and after second heating for the interstitial release protocol. (*a*) Represents the live fluorescence imaging of anaesthetized mice. (*b*) DOX fluorescence intensity signals from the tumor (region of interest) were quantified and expressed as total efficiency. Results expressed as mean ± SEM. * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]



from TTSL treatment in this protocol. However, LTSL treated group survival was not significantly different from the controlled group (treated with HT only). In this protocol, only Lp-Peptide hybrids treated mice showed increased life span (14.9%) compared with other TSL.

### Histopathological analysis

In general, histological analysis showed no signs of tissue damage in any of the groups tested with the exception of TTSL treated mice using the interstitial release protocol. Histological analysis of kidney sections from TTSL group treated with this protocol revealed the deposition of protein within tubular lumen and Bowman's space in the glomeruli. In addition, necrosis of the renal papilla was seen in one animal (1/3 TTSL treated mice; Fig. 5*d*). This is consistent with previous reports of DOX toxicity[35] since the kidney is involved in the excretion of the drug.[36,37] Milder pathological changes were observed in the kidneys of LTSL and Lp-Peptide treated mice with the interstitial release protocol, however these changes were not very severe to cause any clinical or biochemical changes. No other toxicities were found in the heart, liver, spleen and lung sections of all animals.

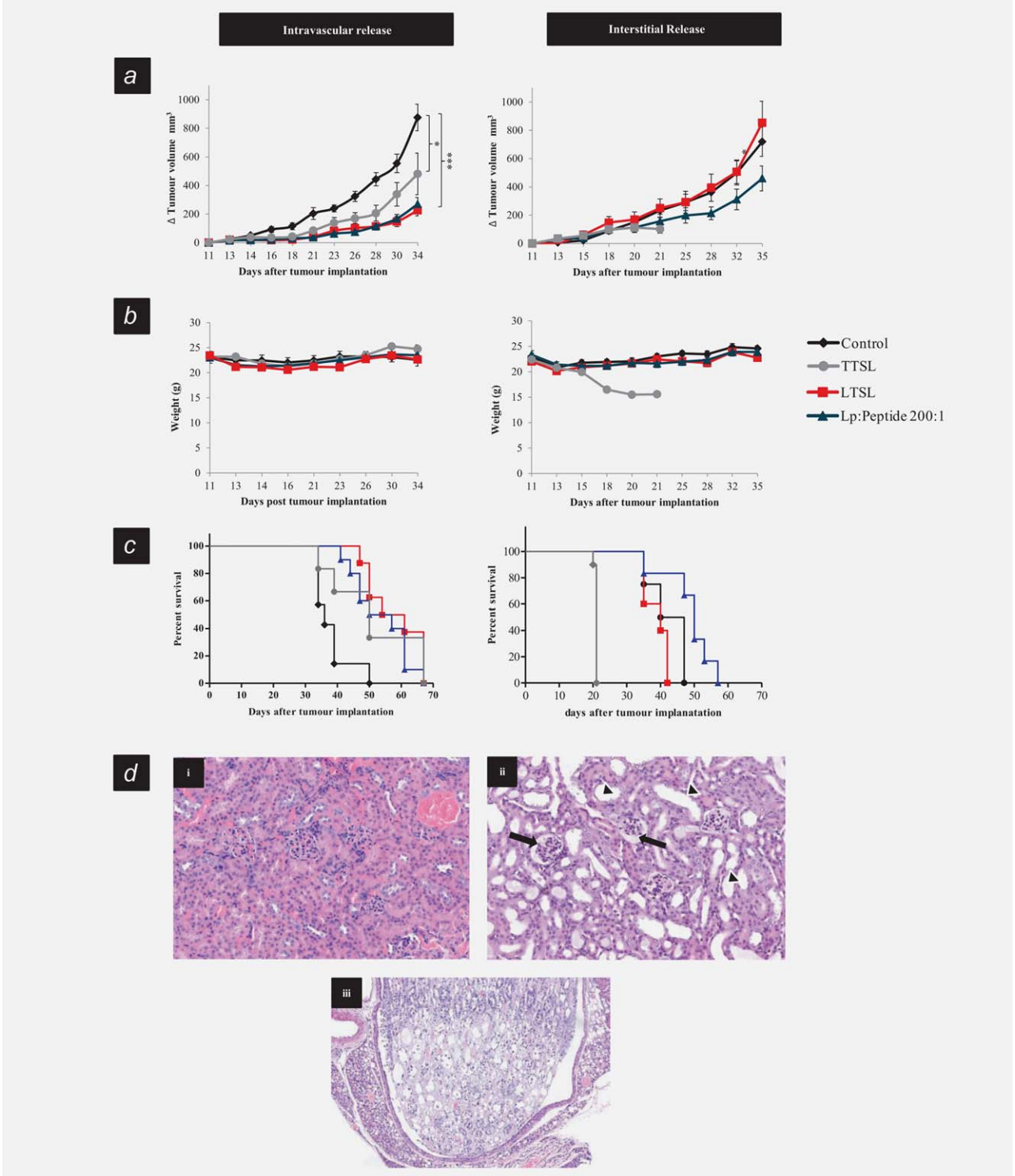



**Figure 5.** ***In vivo*** **tumor growth delay, survival studies and histological analysis.** SW480 tumor-bearing mice were treated with TTSL, LTSL and Lp-Peptide 200:1 liposomes comparing intravascular and interstitial release protocols. (***a***) Change in tumor volume; (***b***) body weight; and (***c***) survival analysis. SW480 ($5 \times 10^6$) cells were injected subcutaneously in the right leg. Therapy started on Day 11 after implantation with average tumor size of 100 $mm^3$. Animals were injected intravenously with TTSL, LTSL and Lp-Peptide 200:1 at 5 mg DOX/kg. Local HT was applied by immersing the tumor-bearing leg into 43 °C water bath. Control animals are noninjected mice treated with HT only. Results expressed as average ± SEM. * indicates $p < 0.05$ and *** indicates $p < 0.001$. (*d*) Histopathological changes in the kidney 10 days after treatment with TTSL (interstitial protocol). H & E staining of kidney tissues from TTSL treated group represent the major pathological changes detected; (i) normal kidney, (ii) pathological changes – arrows demonstrate protein in Bowmans space, arrow heads show dilated tubules with flattened epithelium and (iii) papillary necrosis. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

## Discussion

It has been accepted that the therapeutic activity of long-circulating liposomal anticancer drugs is greatly compromised by the heterogeneous accumulation into the tumor,[38] and the limited bioavailability of encapsulated drug.[39] Enhanced permeability and retention effect (EPR) has always been considered crucial in the liposomal tumor accumulation in animal tumor models,[40–42] however, its clinical benefits are not yet conclusive.[43] Triggered release of liposomal drug content by local mild HT is a promising approach to improve the therapeutic effect of liposomal anticancer drugs. Localised mild HT improves liposomal drug bioavailability,[8] increases liposomal tumor accumulation[7] and offers a new paradigm for (intravascular) drug delivery when combined with ultrafast-release TSL.[20]

Despite the great progress witnessed in the field of TSL over the last 30 years, only few preclinical studies justified the choice of the HT protocols that may be applied clinically and the scheduling between liposome injection and tumor heating based on the matching of physicochemical properties of the TSL, blood profile and tumor accumulation. In most of these studies HT was applied directly after injection[8,10,24,44–47] or shortly after (1–3 hr) while the TSL were still in circulation (aiming at intravascular drug release).[14,45] Only few studies described the use of HT in combination with liposomes after their accumulation into the tumor (interstitial release).[14,27]

LTSL (ThermoDOX®) is an example of TSL that is currently in clinical trials.[25] LTSL system acts by triggering intravascular drug release with mild HT,[24] and double drug penetration depth into the tumor interstitial space compared with free DOX and NTSL (Doxil®).[20] A Phase III trial in liver cancer in combination with radiofrequency ablation has so far failed to show sufficient evidence of clinical effectiveness.[48] Although the results are still being analysed, preclinical studies suggested that the timing between injection and heat application is the most critical factor in the success of this type of TSL due to the possibility of DOX leakage from ThermoDox® into the blood circulation even before HT application[34]and the short blood kinetics of encapsulated drug (only 1.3 hr), which restrict the time frame of the heating protocol.[32,49] The impact of the sequence between LTSL injection and HT treatment on the therapeutic efficacy achieved from intravascular drug release has been illustrated previously in preclinical studies by Ponce *et al.*[23] In a rat fibrosarcoma model injected with LTSL into a prewarmed tumor (injection during HT) the optimum amount of DOX delivered to the tumor and its therapeutic effectiveness were shown.[23] Almost double drug concentration in the tumor was achieved with this protocol compared with injection before HT and associated with greater antitumor effect as evidenced by longer time of tumor progression (34 days for LTSL injected during HT compared with 18.5 days for LTSL given before HT). In another study, Kong *et al.* compared the therapeutic effects of intravascular drug release from TTSL, LTSL and NTSL applying 1 hr HT at 42 °C immediately after injection using a FaDu model (human squamous cell carcinoma xenograft). DOX-loaded LTSL liposomes demonstrated improved therapeutic efficacy compared to TTSL and NTSL liposomes.[8] LTSL-DOX treatment with HT showed complete tumor growth regression 51 days after treatment compared to only 30–35 days from TTSL and NTSL. This increase in therapeutic efficacy is attributed to the amount of DOX tumor accumulation which was found to be 25 ng DOX/mg tissue from LTSL compared to only 7–8 ng DOX/mg tissue from TTSL and NTSL.[8] However, the result of this study was restricted to the first hour after injection only.

As alternative to intravascular drug release, Gaber *et al.* described previously the use of HT with TSL to trigger interstitial DOX release from the extravasated long-circulating TTSL by heating the tumor 24 hrs after injection. However, this study did not show the effect of interstitial tumor drug release from TTSL on therapeutic activity.[14] This has been shown recently by Li *et al.* reporting that use of a two step-HT protocol with slow-releasing TSL (sTSL) to trigger intestinal drug release after tumor accumulation can offer comparable drug tumor levels to that obtained from one-step HT triggering intravascular Dox release from fast releaseTSL (fTSL). In spite of the tumor growth delay in human BLM melanoma, this work showed that a two-step HT protocol combined with sTSL was not as effective as intravascular Dox release from fTSL using a one-step HT.[27]

Our goal in this study was to define the parameters that can affect the choice between intravascular and interstitial triggered drug release to achieve ultimate efficacy of HT-assisted treatment of the Lp-Peptide hybrid system compared to other TSL treatments. The rationale of using 24 hr time point for the second hyperthermia in the interstitial approach is based on the increase tumor vasculature permeability that lasts for few hours after the first hyperthermia,[28] leading to increased liposomes extravasation over that time period. Previously, we have shown that LTSL injection in combination with mild HT offers the highest tumor DOX levels in the 1 hr after HT compared with non-HT conditions expected from the intravascular drug release of LTSL.[30] In contrast, in the case of long circulating TTSL the enhancement in liposome extravasation was observed 24 hr after injection even though the tumor was mildly heated only during the first hour. This was indicated by the increase in tumor levels of both lipids and DOX, which suggested the suitability of TTSL for interstitial triggered drug release after tumor accumulation.[30] In another study, we also have shown that Lp-Peptide hybrids in combination with HT gave high DOX tumor levels 1 hrs after injection equivalent to LTSL DOX tumor levels. In addition, Lp-Peptide TSL showed a significant long term (threefold) increase in DOX tumor accumulation 24 hr after HT compared with LTSL.[29] These findings suggested that Lp-Peptide hybrids could be used for both intravascular and interstitial triggered drug release, which was the aim of investigation in this study.

*In vivo* therapeutic efficacy studies were performed using the SW480 tumor model rather than B16F10 because of the rapid growth and aggressive nature of B16F10 tumor model that makes comparison between the treatment groups challenging, especially that our experiments were designed on single and not multiple injection regimes. Previous reports have shown that tumor drug concentration correlated directly to therapeutic efficacy[8,50,51] and *in vivo* optical imaging, therefore, IVIS imaging studies were performed to correlate tumor DOX accumulation with growth retardation and survival studies.

As descried earlier, Ponce *et al.* showed that the maximum therapeutic activity of LTSL can be achieved by intravascular drug release after injection into preheated tumors. In our study, intravascular drug release was studied by applying HT immediately after injection in consistence with the protocols used in our previous studies.[29,30]LTSL treated animals using intravascular release protocol showed pronounced tumor growth retardation and prolonged survival compared with the control because of their fast drug release that resulted in the highest tumor DOX level immediately after HT. However, LTSL treatment with interstitial release protocol did not show any improvement in tumor growth retardation and survival, which was predicted from its limited tumor DOX accumulation. The leakage of most of the encapsulated drug from LTSL in the circulation before accumulation in the tumor; significantly reduce the fraction of bioavailable drug that reached the tumor.

In the case of TTSL treatment using the intravascular protocol, tumor growth retardation and survival were better compared with control. However, the therapeutic effect was less pronounced compared to LTSL treatment because of their intermediate drug release rate in response to HT. Although TTSL treatment with the interstitial release protocol showed the highest DOX accumulation among the three types of TSL tested, as expected from their long blood circulation time and drug retention, this was also accompanied by severe weight loss and resulted in 50% reduced survival. The rapid toxicity profile observed with the TTSL treatment using the interstitial protocol suggested toxicities, other than cardiotoxicity, are involved, since the cardiotoxicity of DOX is mainly a cumulative effect.[52] This agrees with the findings of Allen *et al.* where they observed similar toxicity profile (gastrointestinal as an example) from mice treated with DOX-loaded DPPC/POPC:CHOL:DSPE-PEG (2:1:0.1) liposomes which has intermediate release properties.[52] Histopathological analysis of organs pooled from TTSL treated mice with interstitial release protocol showed significant pathological changes in the kidneys, which were consistent with findings previously reported with free drug.[35] The possible explanation of the above is the intermediate drug retention of TTSL and their longer blood circulation time when the interstitial protocol was used. This discrepancy in blood profile of TTSL between the two protocols is believed to be due to the release of DOX from TTSL at the tumor site when the HT is applied simultaneously after injection.[44] Similar observations have been reported before by Mayer *et al.* and Allen *et al.* and showed that relatively prolonged blood circulating liposomes with intermediate release rate had higher toxicity compared with those with fast and slow release.[53,54]

In the case of treatment with the Lp-Peptide hybrids, the intravascular protocol showed equally effective tumor growth retardation and survival to that of LTSL treatment. The interstitial release protocol also showed good therapeutic response with Lp-Peptide hybrids. Almost 15% increase in life span was observed, with no signs of toxicity; however, this was not as effective overall as the therapeutic effect observed in the intravascular release protocol.

Interestingly, higher DOX accumulation was achieved from intravascular release compared to interstitial release protocols for the three types of TSL tested. This can be understood based on the advantageous effect of HT on the tumor accumulation of liposomes as well as the triggering of DOX release. Despite the well-known effect of HT on increasing liposomal extravasation into the tumor that can last up to 6–8 hrs after stopping HT, this effect decays over time.[8,26] Maximum increase in nanoparticles extravasation can be achieved when administered with HT due to the contribution from increased in blood flow and triggered local release of DOX from TSL.[8,55]

## Conclusions

This study demonstrated that the drug release capability of TSL is not the sole parameter that determines their therapeutic activity. Protocol design that includes timing of heating and injection of vesicles that should match their physicochemical properties and pharmacokinetic profile also play a pivotal role to the therapeutic effectiveness, as well as the toxicity of TSL. Lp-peptide constructs and LTSL were equally effective and more efficacious than TTSL following the intravascular approach. This study highlights the importance of understanding the properties of newly developed temperature-sensitive systems and correlating them with the appropriate heating regime to maximise their efficacy and advise their further development in the clinical setting.

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## Financial Interests

The authors declare no competing financial interests.

## Supporting Information

Supporting data that includes: biocompatibility of Lp-Peptide Hybrids and *in vitro* cytotoxicity studies; are available free of charge via the Internet.

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