Real-time fluorescence imaging for visualization and drug uptake prediction during drug delivery by thermosensitive liposomes

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Objective: Thermosensitive liposomal doxorubicin (TSL–Dox) is a promising stimuli-responsive nanoparticle drug delivery system that rapidly releases the contained drug in response to hyperthermia (HT) (>40 °C). Combined with localized heating, TSL–Dox allows highly localized delivery. The goals of this study were to demonstrate that real-time fluorescence imaging can visualize drug uptake during delivery, and can predict tumor drug uptake.

Methods: Nude mice carrying subcutaneous tumors (Lewis lung carcinoma) were anesthetized and injected with TSL–Dox (5 mg/kg dose). Localized HT was induced by heating tumors for 15, 30 or 60 min via a custom-designed HT probe placed superficially at the tumor location. In vivo fluorescence imaging (excitation 523 nm, emission 610 nm) was performed before, during, and for 5 min following HT. After imaging, tumors were extracted, drug uptake was quantified by high-performance liquid chromatography, and correlated with in vivo fluorescence. Plasma samples were obtained before and after HT to measure TSL–Dox pharmacokinetics.

Results: Local drug uptake could be visualized in real-time during HT. Compared to unheated control tumors, fluorescence of heated tumors increased by 4.6-fold (15 min HT), 9.3-fold (30 min HT), and 13.2-fold (60 min HT). HT duration predicted tumor drug uptake (p = .02), with tumor drug concentrations of 4.2 ± 1.3 μg/g (no HT), 7.1 ± 5.9 μg/g (15 min HT), 14.1 ± 6.7 μg/g (30 min HT) and 21.4 ± 12.6 μg/g (60 min HT). There was good correlation (R² = 0.67) between fluorescence of the tumor region and tumor drug uptake.

Conclusions: Real-time in vivo fluorescence imaging can visualize drug uptake during delivery, and can predict tumor drug uptake.

Introduction

Conventional cancer chemotherapy has been limited by insufficient drug delivery to the tumor site, with only ~0.1% of the infused drug reaching tumors [1]. To enhance tumor drug uptake, numerous drug delivery systems such as liposomes and other nanoparticles have been devised, most often based on increased tumor nanoparticle uptake due to the enhanced permeability and retention (EPR) effect [1,2]. Even though such drug delivery systems facilitate increased (~0.7% of infused dose [1]) tumor accumulation compared to free (unencapsulated) drug, this did not translate to enhanced tumor control, with patient benefit limited to reduced toxicities. The lack of efficacy improvement is likely because the drug remains encapsulated and does not become bioavailable following tumor uptake [3,4].

Stimuli-responsive delivery systems such as thermosensitive liposomes (TSL) are a promising approach to locally enhance drug bioavailability while also reducing toxicities [5,6]. TSL rapidly release the contained drug in response to hyperthermia (HT) >40 °C, with newer TSL formulations based on the intravascular triggered release paradigm; here, drug release from TSL occurs within the heated microvasculature, followed by rapid tissue uptake of the released drug [3,7,8].

While various drugs have been encapsulated in TSL, doxorubicin (Dox) is the most widely investigated agent [5,6]. Combined with localized heating, TSL–encapsulated doxorubicin (TSL–Dox) allows highly localized delivery (~10–30× local dose compared to unencapsulated Dox) [6,9,10], with complete regression of tumors achieved in...
some preclinical studies [9,11,12]. Only a single formulation of TSL-Dox has so far advanced to clinical trials in conjunction with either radiofrequency ablation (RFA), high intensity focused ultrasound (HIFU), or microwave HT, for treatment of liver cancer and recurrent chest wall cancer [13–18].

The drug quantity delivered by TSL depends on the heating device, as different devices generate different heating profiles [19]. In addition, the duration of HT dictates drug uptake [20,21]. Both the duration and method of HT in preclinical animal studies differ considerably, with HT duration ranging from 2 to 12 min for RFA [12,20], 2 to 40 min for HIFU [22,23], 60 min for heated water bath [8,9,24] and cold light lamp [19], and 5 to 60 min when a laser was used as heat source [19,25].

In the current study with TSL–Dox, we present real-time in vivo fluorescence imaging methods for monitoring, and for quantification of tumor drug uptake. Dox is inherently fluorescent, and in vivo microscopy studies have exploited this property to monitor drug uptake at the microscopic level during delivery with TSL–Dox and to characterize drug penetration [8,26–28]. One study employed optical spectroscopy measurements obtained via a fiber-optic probe to quantitatively monitor tumor drug uptake [29]. Several groups have employed whole-body in vivo fluorescence imaging to visualize drug accumulation from TSL–Dox release after completion of HT. To our knowledge, there is no in vivo whole-body fluorescence imaging study that monitored spatial drug distribution in real-time during heating and delivery. The goals of this study were to demonstrate that (1) time lapse in vivo fluorescence imaging can be used to monitor drug uptake in real-time during HT mediated drug delivery, and (2) that in vivo fluorescence intensity can predict tumor Dox accumulation (Figure 1).

**Materials and methods**

**Tumor cell line culture**

Lewis lung carcinoma (LLC) cells were routinely cultured at 37 °C in 5% CO₂/95% air in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich, D6429) supplemented with 10% inactivated fetal bovine serum (Invitrogen), 1% Penicillin and streptomycin (Invitrogen). TSL–Dox has relevance for lung cancer since tumor ablation is used in lung cancer, and the combination of tumor ablation and TSL–Dox is currently in clinical trial for liver cancer [13,30]. In addition, there are prior studies with TSL–Dox in LLC tumors [27].

**Mice and tumors**

All experiments were approved by the Medical University of South Carolina’s Institutional Animal Care and Use Committee. 8–12 week old female athymic BALB/c nude mice were housed at 3–5 mice in ventilated polycarbonate microisolation cages. Each cage contained sterile 1/8” paper strip enrichment (Enviro-Dri, Shepherd Specialty Papers, Milford, NJ, USA) and 1/8” corn cob bedding (The Andersons, Maumee, OH, USA). All mice had free-choice access to autoclaved reverse osmosis water and commercial chow (Teklad Global Diet 2918S, Envigo, Madison, WI, USA). The rodent housing rooms were maintained at 22 ± 1 °C on a 12:12 h light:dark cycle. All animals were acclimated for at least 5 days prior to any experimental manipulation. A specific-pathogen-free status was maintained for Mycoplasma pulmonis, Helicobacter spp., endo- and ecto-parasites, mouse hepatitis virus, minute virus of mice, mouse parvovirus, enzootic diarrhea of infant mice virus, ectromelia virus, Sendai virus, pneumonia virus of mice, Theiler murine encephalomyelitis virus, reovirus, lymphocytic choriomeningitis virus, not specified
mouse adenovirus, mouse norovirus, and polyomavirus. Tumors were initiated per procedures previously described [31]. Briefly, 1 × 10^6 LLC cells were injected subcutaneously to the lower leg flanks of the animal on both sides. The tumors were allowed to grow until they reached a diameter of 4–5 mm before being treated.

**Thermosensitive liposomal doxorubicin (Dox) preparation**

Thermosensitive liposomal doxorubicin (Dox) was prepared as previously described by Negussie et al. [32] with slight modifications. Briefly, lipids, dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-PEG2000 (DSPE-PEG2000), and monostearoyl phosphatidylcholine (MSPC) (Avanti Lipids, Alabaster, AL, USA) were dissolved in chloroform and then dried under a stream of air to form a thin film of lipids. This thin film was hydrated with 300 mM citrate buffer (pH 6.5) and then dried under a stream of air to form a thin film that could be used in the experiments.

**Hyperthermia probe**

Our goal was to perform fluorescence imaging during heating. Since most HT devices are not sufficiently small for use inside the imaging system, we custom-designed a miniature HT probe. This HT probe for heating subcutaneous tissues was based on a 2.5 mm diameter thermistor (Honeywell, NTC 121). The uninsulated wires attached to the thermistor element were insulated by inserting them in glass capillary tubes, which were then filled with epoxy resin (Figure S1(A)). We developed hardware and software to heat the probe to a defined target temperature. Part of the software was implemented on the Arduino prototyping platform, and handled measurement of heating probe resistance as well as applying a voltage to the thermistor probe. The Arduino board communicated with a PC via software written in Python that ran on the PC. The Python software read the thermistor resistance, converted it to temperature, and then calculated the required thermistor voltage based on a proportional–integral (PI) controller so that the thermistor achieved the desired target temperature. A graphical user interface allowed the user to enter the target temperature and monitor the probe temperature in real-time (Figure S1(B)). The thermistor was calibrated to determine the relation between temperature and thermistor resistance by placing the thermistor probe in a water bath of defined temperature. To estimate the expected temperature profile produced by the probe in vivo, we measured the temperature profiles produced by the probe in gel phantoms at 50 °C target temperature while recording temperature maps with an infrared camera (Mikron M7500) for 60 min. In addition, we measured surface skin temperature profiles produced by the probe in live animals inside the fluorescence imaging system with an infrared camera (FLIR C2).

**Thermal animal support**

We performed studies to ensure that the core body temperature of the animals stays within the physiological range during HT and imaging, to determine the optimal temperature setting for thermal support inside the imaging system. We set the temperature of the thermal support in the imaging system to either 34°C or 37°C while measuring the rectal temperature of the animals for 1 h inside the imaging system (n = 3 per group). In addition, we obtained blood samples at 6 min and at 75 min after injection of TLS-Dox to quantify plasma Dox concentration and identify any accelerated leakage from TLS due to elevated body temperature.

**Temperature sensitivity of doxorubicin fluorescence**

Since fluorophores often exhibit temperature dependence of fluorescence intensity, we performed an in vitro study to quantify any temperature sensitivity of Dox fluorescence. A Dox solution was pumped into a capillary tube placed on top of a heating element as used in prior studies [33]. Fluorescence images were acquired at temperatures between 37 and 50°C and Dox fluorescence intensity was quantified.

**In vivo fluorescence imaging**

On the day of the experiments, animals were weighed and tumor sizes were measured. Mice were anesthetized by isoflurane (1–5%), a background fluorescence image was obtained, and TLS-Dox was intravenously administered at a dose of 5 mg/kg. Five to 6 min after the infusion of TLS-Dox, a plasma sample was collected and 200 μL of Lactated Ringer’s solution was administered subcutaneously for hydration. The animals were then transferred to the in vivo fluorescence imaging system (Maestro, Caliper Life Sciences, Hopkinton, MA, USA). The thermal support for the animal was set at 34 °C rather than 37 °C to keep the animals body temperature within physiological range and avoid any enhanced systemic leakage of TLS-Dox. Care was taken to ensure that the heating probe was on the skin surface located centrally on the tumor. We employed a fixed angle standoff to stabilize the probe and ensure a similar probe angle in all animals. In addition, care was taken to ensure the same...
pressure was applied. The contralateral tumor was used as a control without being exposed to HT. The temperature probe was set at 50 °C, which resulted in a ~43 °C probe surface temperature (50 °C is measured inside the probe center). The tumors were exposed for 15, 30 or 60 min to HT. Time-lapse whole-body fluorescence images were obtained before start of HT, every 5 min while heating, and until 5 min after heating was stopped. Then the heating probe was removed, and a final fluorescence image was taken. If imaging lasted for over 30 min, the animals were given 50 μL of Lactated Ringer’s solution subcutaneously every 30 min to keep them hydrated. Following imaging, the animals were sacrificed, blood was collected, and the entire tumors were harvested. Plasma samples obtained by centrifuging the blood samples and the harvested tumor tissues were stored at ~80 °C until analyzed. Extracted tumors were imaged by the fluorescence imaging system before being frozen.

Fluorescence imaging was performed with filters appropriate for Dox for excitation (523 nm) and emission (610 nm). The images were exported from the imaging system software as tif-format files and imported into ImageJ (NIH, Bethesda, MD). In ImageJ, image stacks were created from all acquired images during each individual HT experiment. Images were then converted from 16- to 32-bit to improve accuracy of any subsequent image processing. The brightness and contrast of the entire image stack was adjusted to ensure optimal visualization of tumor fluorescence. The fluorescent region of the heated tumor was manually outlined (region of interest (ROI)), excluding the thermistor (heating probe). The average intensity of the tumor ROI was calculated, and intensity of the background image (before heating) within this ROI was subtracted for each image frame. For the control tumors, a tumor ROI of equivalent size to those of heated tumors was drawn at the contralateral tumor location.

**Plasma Dox quantification**

Plasma Dox concentration was measured by a fluorimeter as described previously [36]. Briefly, samples were thawed. Subsequently, to 30 μL of plasma sample, 90 μL of phosphate buffered saline and 100 μL of 10% Triton™X-100 (diluted in deionized water) were added. The fluorescence intensity of the sample was measured by a microplate reader (Synergy HT, Biotek Instruments Inc., Winooski, VT, USA) using appropriate filters for Dox (excitation 485 nm, emission 590 nm). The drug concentration was determined by a comparison against a standard curve prepared from mouse plasma samples spiked with known concentrations of mouse Dox (1–100 μg/ml).

**Tumor tissue Dox quantification**

Doxorubicin quantification of the entire tumor tissue samples was performed following a protocol reported by us earlier [37]. Tumor tissue samples were homogenized in aqueous KH₂PO₄ (pH = 3.8) using a homogenizer (Bead Ruptor 24; Omni International, GA, USA) at 5 m/s for five minutes. The homogenized tissue was centrifuged, and supernatant collected. A 90 μL fraction of each extracted sample was mixed with 50 μL, 2.5 μg daunorubicin/mL (internal standard (IS)) in a 2 mL vial, vortexed and incubated at 37 °C for 15 min. Acetone (250 μL) and a saturated solution of zinc sulfate (100 μL) were added, and samples were vortexed and incubated at 37 °C for 15 min, centrifuged, and 200 μL of supernatant recovered and dried. Dried residues were dissolved in high-performance liquid chromatography (HPLC) mobile phase (acetonitrile and deionized water [34:66] with 0.1% trifluoroacetic acid) and Dox concentration was evaluated by using a reversed-phase column (Zobrax Eclipse Plus C18; Agilent Technologies, CA, USA). Detection was performed at appropriate excitation/emission wavelengths of 498/593 nm. Data acquisition was done using ChemStation Version B 04.03 software (Agilent Technologies). Concentrations of the analyte in tissue samples were determined using peak-area ratios of the sample analyte to the IS from the linear calibration curve using least squares regression method based on the nominal concentration (generated from untreated tissue spiked with different concentration of Dox and IS). Additional details are available as Supplementary Information.

**Statistical analysis**

To statistically validate the uptake of Dox in tumor tissue, we ran several non-parametric tests. First, we compared drug concentration before and after HT (n = 15 pairs) using the Wilcoxon signed rank test. To determine if the uptake of Dox was associated with duration of HT, Wilcoxon sum rank tests were used to compare the means of the three different HT treatment durations (15 min vs. 30 min vs. 60 min). Additionally, a linear regression model was constructed regressing Dox concentration in the tumor on the length of HT with measurements from unheated control tumors assigned a treatment time of zero minutes. To determine if fluorescence and Dox concentration were associated, a linear regression model was built. Additionally, linear regression models were created to evaluate the associations between HT duration, Dox concentration, and tumor ROI fluorescence (AU).

**Results**

**In vitro characterization of TSL–Dox**

Complete release of Dox from the TSL–Dox occurred within 2 s at temperatures above 40 °C (Figure S2(a)). About 20% of the drug was released at 37 °C within the first few seconds [36] (data not shown). The liposome size was 109 ± 4.9 nm based on dynamic light scattering (Figure S2(b)).

**Temperature sensitivity of Dox fluorescence**

Compared to 37 °C, fluorescence intensity of Dox increased by 6% when heated to 40 °C, and by 11% when heated to 43 °C (Figure S4).
Hyperthermia probe

Gel phantom experiments showed that temperature reached steady-state within ~2 to 3 min (Figure 2(a)). While the HT probe temperature was set to 13 °C above phantom baseline temperature, this represents the internal probe temperature (i.e., inside the heated thermistor bead). The probe surface temperature reached only 6 °C above baseline, which would correspond to 43 °C in vivo (37 ± 6 °C). Temperature dropped rapidly with distance from the probe tip, and temperatures adequate for TSL drug release were limited to ~1 to 1.5 mm distance from the probe (Figure 2(b)).

In addition, we performed in vivo studies inside the imaging system with the same environmental temperature settings as used in in vivo studies with TSL–Dox where we measured tumor surface temperature with an infrared camera (FLIR C2, FLIR Systems) during heating in two mice, where we assumed an emissivity of 0.98 for mouse skin [38] (Figure 3(c)). Steady state was reached within 5–10 min. Since TSL release effectively above ~40 °C, we considered the region within the 40 °C isotherm as an estimate of the region of drug release. The diameter of the tissue region above 40 °C was 4–4.6 mm after 1 min of heating, and plateaued at 4.6–5.4 mm after 5–10 min (Figure 3(d)). This corresponds to a location of the 40 °C isotherm at a distance of 1.1–1.5 mm from the thermistor probe surface, similar to the results from the gel phantom studies. Upon cessation of HT, temperature returned to baseline within less than 1 min.

Dox uptake can be visualized by fluorescence imaging during delivery

Following infusion of TSL–Dox, there was an overall increase in the fluorescence compared to pre-infusion in the whole animal. The fluorescence of the unheated tumors did not change significantly during HT exposure of the contralateral tumors. The uptake of Dox by tumors exposed to HT could be monitored by fluorescence imaging during heating, visualizing spatially where drug was delivered (Figure 3(a)). The fluorescence intensity increased as long as tumors were exposed to HT, suggesting continuous drug uptake as long as HT was applied. Once the heating stopped there was a rapid decrease in the fluorescence suggesting wash-out of free Dox not bound to tissue constituents (Figure 3(b)). HT duration was a statistically significant predictor of tumor ROI fluorescence ($p = .03$). Compared to unheated control tumors, tumors exposed to HT had fluorescence of the tumor ROI enhanced by 6.2-fold (15 min HT), 8.8-fold (30 min HT) and 9.7-fold (60 min HT) (Figure 3(d)). The reported fluorescence intensity in Figure 3(b) is limited to tumor ROI regions not visually obstructed by the HT probe.
Five minutes after the conclusion of HT treatment, the HT probe was removed (Figure 3(c)) and mean fluorescence intensity was measured of the now unobstructed tumor ROI (Figure 3(d)). Compared to unheated control tumors, the fluorescence intensity in tumor ROIs of heated tumors was enhanced 4.6-fold (15 min HT), 9.3-fold (30 min HT) and 13.2-fold (60 min HT) (Figure 3(d), Table 1). While during heating (Figure 3(a,b)) the temperature sensitivity of Dox contributed towards the observed increase in fluorescence (only 15% of the observed increase can be attributed to enhanced fluorescence at hyperthermic temperatures (see Figure S4)), the imaging studies after conclusion of HT (Figure 3(c,d)) were done with tumor temperature returned to baseline as confirmed by our infrared imaging studies, that is, without any contribution from enhanced fluorescence at hyperthermic temperatures.

**Table 1.** Tissue fluorescence and concentration of Dox exposed to varying durations of HT.

| Time Post Infusion | HT | No HT | HT | No HT | HT | No HT |
|-------------------|----|-------|----|-------|----|-------|
| 15 min HT         | 63.2 ± 21.3 | 13 ± 4.6 | 149.5 ± 50.5 | 16.1 ± 4.2 | 169.9 ± 95.3 | 12.9 ± 1.6 |
| 30 min HT         | 149.5 ± 50.5 | 16.1 ± 4.2 | 169.9 ± 95.3 | 12.9 ± 1.6 | 169.9 ± 95.3 | 12.9 ± 1.6 |
| 60 min HT         | 169.9 ± 95.3 | 12.9 ± 1.6 | 169.9 ± 95.3 | 12.9 ± 1.6 | 169.9 ± 95.3 | 12.9 ± 1.6 |

Concentration of doxorubicin is shown as μg/g tumor tissue ± SD. Fluorescence is shown as AU ± SD (n = 5/group).

Duration of HT dictates tumor drug uptake

The excised tumors were imaged by fluorescence imaging both from lateral and medial views, demonstrating that the drug was not evenly distributed throughout the tumors (Figure 4(a)). Measurement of tumor drug concentration by HPLC revealed that compared to unheated control tumors, the tumor Dox concentration of heated tumors was 1.9-fold (15 min HT), 2.9-fold (30 min HT) and 5.2-fold (60 min HT) higher (Figure 4(b), Table 1). Tumor dox concentration was significantly higher with, compared to without HT (Table 2). Based on a linear regression analysis with HT duration (15, 30 and 60 min) as predictor variable, HT duration was a statistically significant predictor of tumor drug uptake ($p = .02$); on average, each additional minute of HT enhanced tumor drug uptake by 0.31 μg/g.

**Plasma pharmacokinetics**

Plasma concentration of Dox was measured by fluorometry, as a prior study showed that HPLC and fluorometry achieve similar accuracy in plasma [36]. Peak Dox plasma concentration was 74.5 ± 22.2 μg/mL, measured 5 min post infusion (Figure 5(a)). Large variation in the concentration of Dox in plasma of different animals was observed, and may be due to differential clearance between animals, from varying blood volumes, and from variation in TSL leakage. Plasma half-life
was 56.0 ± 15.9 min, which is similar to a prior study in mice [39] and comparable to the half-life of 45 min observed in canine studies [36], and 55 min from clinical studies in human patients [13], all with lysolipid based TSL formulations as used here.

**Elevated body temperature results in enhanced systemic TSL leakage**

We measured the rectal temperature of animals during 60 min inside the imaging system with thermal support either at 34 °C or 37 °C \((n = 3\) per group). The body temperature in the animals with 34 °C support was within the physiological range \((36–37 °C)\) for BALB/c mice [40], whereas body temperature was elevated to above 39 °C when thermal support was set to 37 °C (Figure S3). The body temperature dropped by 2–3 °C immediately after the administration of TSL-Dox since the solution was at room temperature, and returned to baseline within ~20 min. The elevated body temperature at 37 °C thermal support setting resulted in premature leakage of drug from TSL. The plasma concentration 6 min after TSL-Dox administration was 59.5 ± 2.2 ug/ml \((34 °C\) thermal support) and 57.3 ± 6.7 ug/ml \((37 °C\) thermal support), and dropped to 38.4 ± 8.7 ug/ml \((34 °C\) thermal support) and 8.3 ± 3.7 ug/ml \((37 °C\) thermal support) after 75 min (Figure 5(b)). These results demonstrate the importance of monitoring of body temperature and selecting an appropriate thermal support temperature in any TSL studies.

**In vivo fluorescence intensity is predictive of tumor drug concentration**

Fluorescence intensity was a statistically significant predictor of tumor drug uptake \((p = .02)\), and there was a strong linear correlation \((R^2=0.67)\) between the fluorescence intensity measured after HT conclusion and the Dox delivered to the
tumors (Figure 6). Since the tumor ROI was manually drawn and not based on the actual tumor boundaries (as tumors were not visible under fluorescence imaging), it is reasonable to assume that the correlation would improve further if we were able to measure fluorescence within the actual tumor region, for example, by using fluorescently labeled tumors.

For our HT probe, the predicted hyperthermic zone (>40 °C) where TSL release occurs extends to about 1.5 mm depth, based on infrared imaging studies in an agar gel phantom (Figure 2). This means that tumors were only partially exposed to hyperthermic temperatures, with some parts not adequately heated. This was confirmed by fluorescence imaging of extracted tumors that showed limited fluorescence when viewed from medial direction (Figure 4(a)). Thus, we anticipate that tumor drug uptake could be further enhanced with a more uniform HT method.

Discussion

Thermosensitive liposomes are a triggered drug delivery system that releases the encapsulated drug upon exposure to hyperthermic temperatures (>40 °C), and were first described four decades ago [41]. The more recent fast-release TSL formulations encapsulating Dox [31,42] or idarubicin [43] are based on the intravascular triggered release paradigm, where drug release occurs inside the heated tumor microvasculature, followed by tumor uptake of the released drug [3,7,8].

In the current study we employed a TSL–Dox formulation similar to the one currently used in clinical trials [31] with slightly modified lipid composition as described earlier [32]. We applied HT locally to subcutaneous tumors by a custom-designed miniature probe (Figure 3(a)). Importantly, the miniature probe enabled us for the first time to perform real-time in vivo fluorescence imaging inside a fluorescence imaging system during HT. Since Dox is fluorescent, the tissue region where drug uptake took place could be visualized during delivery, providing direct feedback on location of delivery (Figure 3(a)). Fluorescence of tissue surrounding the HT probe was visibly enhanced within 5 min, and increased as long as heat was applied. Fluorescence rapidly declined once heating stopped (Figure 3(b)), suggesting that drug delivery was limited to the duration of HT, similar to prior studies [3,8,44]. Since Dox fluorescence is slightly enhanced at elevated temperatures (by 6–11% between 40–43 °C, see Figure S4), a small fraction of the observed fluorescence increase during heating (Figure 3(a,b)) can be explained by this direct temperature dependence of Dox fluorescence. Any persistent fluorescence after tumor temperature returned to baseline (<1 min after HT cessation) is attributable to tumor drug uptake alone (Figure 3(c,d)).

Intravital studies have demonstrated that for TSL based delivery, fluorescence primarily originates from intracellular Dox that has been taken up by cells [44], rather than from intravascular free or encapsulated drug. This suggests that also in our studies, fluorescence comes primarily from intracellular drug.

There are several factors that affect the amount of drug delivered to the targeted tissue by TSL based on the intravascular triggered release paradigm. These factors include the device/method of HT (since the device affects tumor temperature distribution) [19], the applied temperature, and the HT duration [11,20,21]. Prior preclinical studies with TSL employed varying HT durations ranging from 2 to 60 min [8,9,12,19,20,22,24,25], with all studies limited to a single duration except for three recent reports that examined two HT durations [11,20,21]. In the current study we demonstrated that the amount of Dox delivered to tumors quantified by HPLC increased with HT duration, for the three durations of 15, 30 and 60 min (Figure 4(b), Table 1). HT duration was a statistically significant predictor of tumor drug uptake (p = .02), and on average each additional minute of heating increased tumor drug uptake by 0.31 μg/g. In addition to tumor drug uptake, also the mean fluorescence in the tumor ROI after conclusion of heating depended on the HT duration (p = .03) (Figure 3(c,d)). These results suggest that tumor drug uptake can be modulated by adjusting the HT

Figure 6. Fluorescence intensity predicts drug delivered to tumors. The mean fluorescence in the tumor ROI measured after hyperthermia conclusion was predictive of the concentration of doxorubicin delivered to tumors (p = .002).
duration, providing control over the locally delivered dose – this ability to modulate the dose is unlike the vast majority of drug delivery systems that provide no means to adjust this locally delivered dose. The dependence of tumor drug uptake on HT duration can be explained based on the intravascular triggered release paradigm: TSL-encapsulated drug in systemic circulation continuously enters the heated tissue volume, releasing the drug. This process continues as long as heat is applied, and TSL-encapsulated drug is available in systemic circulation. Therefore, the longer heat is applied, the more drug is locally released.

Further of considerable relevance is, whether real-time fluorescence was predictive of tumor drug uptake. We found that mean fluorescence intensity in the tumor ROI at the conclusion of HT (after the HT probe was removed) was predictive of tumor drug uptake \( (p = 0.002) \) and correlated well with the quantity of Dox delivered to the tumor \( (R^2=0.67) \) (Figure 6). This is a particularly promising result since we did not use the exact tumor boundaries to define the tumor ROI where fluorescence was measured, and presumably the correlation would further increase with fluorescence measurement limited to the actual tumor region. Thus, with real-time fluorescence imaging during HT we could monitor where the drug is delivered to, and also predict how much drug was delivered. Combined with the ability to modulate drug uptake based on HT duration, this opens up the possibility of delivery of a prescribed dose to a targeted tissue region in rodent studies. In this scenario, HT would be applied until the fluorescence in the target volume corresponds to the desired minimum dose.

The presented methods are however limited to fluorescent drugs (e.g., anthracycline family, topotecan, methotrexate), and to fluorescently labeled drugs (e.g., cisplatin, carboplatin [45]). Another limitation of our study is, that we only partially exposed the tumors to hyperthermic temperatures. While this does not impact the study conclusions, future studies where tumor control is measured and compared to tumor uptake predicted by fluorescence imaging will require a more effective HT device that can expose the tumor completely to hyperthermic temperatures.

For most human tumors, 3D imaging would be required to ensure adequate drug delivery to all regions within a deep-seated tumor volume which is not possible with fluorescence imaging. Several studies have attempted to estimate the delivered drug dose by indirect methods via co-encapsulating MRI contrast agents together with Dox that are released together with the drug [23,32,46], and recent studies demonstrate very good correlation between MR contrast uptake and tumor drug uptake [47,48]. The described methods are thus more directly applicable to preclinical studies with limited applicability in human tumors apart possibly from surface tumors.

**Conclusions**

We demonstrated that real-time in vivo fluorescence imaging can visualize where drug uptake takes place during delivery with TSL. In addition, in vivo fluorescence was predictive of tumor drug uptake, and we demonstrated that HT duration dictates tumor drug uptake. The combination of these concepts may enable a preclinical platform for delivery of a prescribed dose to a targeted tumor.

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**Disclosure statement**

No potential conflict of interest was reported by the authors.

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