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Ultrasound triggered release of cisplatin from liposomes in murine tumors

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ABSTRACT

The ability of low frequency ultrasound (LFUS) to trigger the release of drugs from nano sterically stabilized liposomes (nSSL) in vitro, without affecting the drugs' chemical integrity or biological potency, has been previously shown. Herein, the ability of LFUS to (a) trigger the release of cisplatin from nSSL in vivo, and (b) affect the therapeutic efficacy by locally releasing the drug, was studied. For this, nSSL loaded with the anti-cancer chemotherapeutic agent cisplatin were injected intraperitoneally (i.p.) to mice bearing well-developed J6456 murine lymphoma tumors in their peritoneal cavity. Then, LFUS was applied externally to the abdominal wall for 120 s, and drug release was quantified. Nearly 70% of the liposomal cisplatin was released in tumors exposed to LFUS, compared to <3% in those not exposed to LFUS. The effect of LFUS-induced localized drug release on the therapeutic efficacy was tested on BALB/c mice with C26 colon adenocarcinoma tumors in a footpad. Mice were injected *intravenously* with nSSL cisplatin, and 24 h later, the tumor was exposed to LFUS. The group treated by liposomal cisplatin combined with LFUS, compared to all other groups (i.e., free cisplatin with or without LFUS, or liposomal cisplatin without LFUS, or LFUS alone, or no treatment) had the best therapeutic score; tumors stopped proliferating and then regressed over time.

This work presents a modality for the release of drugs from liposomes in vivo using LFUS. Implications of these findings for clinical applications of LFUS-induced liposomal drug release are discussed.

1. Introduction

Cisplatin is one of the most widely used agents in the treatment of a variety of solid tumors, particularly genitourinary, head and neck, and lung tumors [1–4]. However, the clinical use of cisplatin has been limited both by its toxicity, particularly nephrotoxicity and cumulative neurotoxicity, and by the emergence of intrinsic and acquired resistance in many common tumor types. A variety of drug delivery strategies to circumvent cisplatin-related toxicity, such as microspheres [5], nanoparticles [6], unilamellar or multilamellar liposomes [7,8], and polymeric micelles [9] have been reported, with varying results.

Stealth cisplatin is a formulation of cisplatin encapsulated in nano sterically stabilized liposomes (nSSL); these have a prolonged circulation time [10] and are highly effective in targeting tumors [11] and inflamed tissue [12], presumably due to "leakage" of the nSSL through compromised tumor and inflammation vasculature [13,14]. Unlike other nSSL encapsulated drugs, such as Doxil™ for cancer treatment [15], nSSL-encapsulated methylprednisolone hemisuccinate for rheumatoid arthritis treatment [16], that have high therapeutic efficacy, Stealth cisplatin showed poor therapeutic efficacy. Bandak et al. [17], using three murine tumor models, showed that despite the fact that administered nSSL cisplatin had prolonged circulation time and enhanced tumor uptake, poor therapeutic efficacy resulted because of very low release kinetics of cisplatin from the liposomes. Similar low levels of release were shown by Zamboni et al. [18,19] after administration of Stealth cisplatin to mice bearing melanoma tumor models. Another study, by Vail et al. [20], showed that it was safe to administer high doses of Stealth cisplatin to dogs with spontaneously arising osteosarcoma. However, in this case, increasing the dose did not result in an improvement in the therapeutic efficacy [20]. These data suggest that cisplatin was not released from the liposomes at a sufficient rate for suitable bioavailability [21,22]. Meerum et al. [23] and Veal et al. [24], investigating the pharmacokinetics of Stealth cisplatin in humans, came to the conclusion that cisplatin fails to release from the liposomes in vivo.

Ultrasound is used in the clinic for a wide range of applications: imaging, flow analysis, physiotherapy, tumor and fibroid ablation, kidney-stone shattering, and others. The ability to induce heating

Abbreviations: LFUS, low frequency ultrasound; SUV, small unilamellar vesicle; GPC, gel permeation chromatography; AAS, atomic absorption spectroscopy; nSSL, nano sterically stabilized liposomes.

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and/or mechanical effects by ultrasound has been used to release drugs from different types of carriers [25,26], such as polymeric assemblies [27], temperature-sensitive and temperature nonsensitive liposomes [28], micelles [27,29] and drug delivery systems which act also as contrast agent carriers [30,31]. Low frequency ultrasound (LFUS), specifically, has been used to enhance the permeability of biological membranes [26,32–35]. LFUS also increases the permeability of liposomes, which have a phospholipid bilayer similar to that of biological membranes [36]. A previous study, conducted in our group, showed that LFUS is more effective than higher frequency (1- and 3-MHz) ultrasound in releasing doxorubicin from liposomes [37]. Lin and Thomas [38] showed that LFUS is capable of releasing an encapsulated dye from liposomes in vitro. Another study, by Myhr and Moan [39], suggested that there was a synergistic therapeutic effect when mice with human colon cancer cell tumors were treated by combined therapy of liposomal doxorubicin followed by exposure to LFUS. However, the reasons for this synergism and its correlation with triggering drug release were not studied.

In a previous study [36] we showed that exposing Stealth cisplatin to LFUS at an intensity of 3.3 W/cm^2 for different periods of time (30 to 180 s), resulted in a time-dependent release, reaching ~62% after 180 s of LFUS irradiation. The chemical integrity and biological potency of the drug were not affected by LFUS [36]. Analyzing the drug release data showed that LFUS-triggered cisplatin release follows first order kinetics [36]. The proposed mechanism of LFUS-induced liposomal drug release is the formation of transient pore like defects in the liposome membrane, through which the drug is rapidly released [36].

The aims of this study were to investigate (a) whether it is possible to release a drug from its liposomal carrier at a tumor site and (b) to determine whether such release will have a beneficial therapeutic effect.

Our research approach was to inject a nSSL-encapsulated drug, cisplatin, which is known to have extremely low release kinetics [17–24], either directly to the tumor site, for quantifying release, or intravenously (i.v.), enabling passive targeting and accumulation at the tumor site [14,40], for evaluating the therapeutic benefit. After nSSL administrations, the area in the body with the lymphoma or C26 tumor was immersed in water (acting as a coupling medium), and exposed to ultrasound by bringing the probe in close proximity to the skin above the tumor. The ultrasonic output intensity was held constant throughout the experiments. It may be assumed that the actual LFUS exposure intensity at the tumor site is slightly lower than at the source due to ultrasound dissipation. The levels of drug release and tumor proliferation were studied.

2. Materials and methods

2.1. Liposomes

Stealth™ cisplatin, generously provided by ALZA (Mountain View, CA), consists of sterically stabilized nano liposomes (nSSL) <100 nm in diameter, having a lipid composition of 51 mol% hydrogenated soybean phosphatidylcholine (HSPC), Mw 750 (having an iodine value of 3.0); 5 mol% polyethyleneglycol distearoyl-phosphoethanolamine (m^{2000}PEG DSPE), Mw 2774; and 44 mol% cholesterol. These liposomes were prepared and passively loaded with cisplatin, as described by Peleg-Shulman et al. [41], to achieve a drug-to-phospholipid mole ratio of 0.058. Stealth cisplatin was supplied as a colloidal dispersion of 1 mg cisplatin/mL (57.5 mM phospholipid) in an isotonic solution of 10% w/v sucrose, 1 mM sodium chloride and 10 mM histidine buffer, pH 6.5.

Nano liposomes, 100 nm in diameter, radiolabeled with ^{3}H-cholesteryl hexadecyl ether (CE, Perkin Elmer), 0.44 mol%, (0.5 μCi/μmol total phospholipid) were prepared at a total phospholipid concentration of 30 mM as previously described [12].

Liposome size distribution was measured by dynamic light scattering (DLS) using an ALV NIBS/HPPS particle sizer equipped with an ALV 5000/EPP multiple digital correlator, at a scattering angle of 173° (ALV, Langen, Germany).

2.2. In vivo models

All in vivo experiments were conducted either in the animal facility of the Shaare Zedek Medical Center or of the Hebrew University-Hadassah Medical School, Jerusalem, under specific pathogen free (SPF) conditions, and were reviewed and approved by the Institutional Animal Care and Use Committee of the Hebrew University-Hadassah Medical Center.

2.2.1. Triggered release of cisplatin in J6456 murine lymphoma tumors

2.2.1.1. Tumor model. Approximately one million J6456 murine lymphoma cells suspended in 200 μL of phosphate buffered saline (PBS, Biological Industries, Beit Haemek, Israel), pH 7.4, were injected i.p. [42] into thirteen 8 week old BALB/c female mice (Harlan Laboratories, Ltd., Jerusalem, Israel). Two weeks after cell inoculation, abdominal swelling due to ascites formation was observed, and animals were divided into three test groups: (i) control (placebo), two mice; (ii) Stealth cisplatin without LFUS, five mice; and (iii) Stealth cisplatin plus LFUS, six mice.

2.2.1.2. Drug treatment. In groups ii and iii, nSSL cisplatin at a dose of 15 mg drug (50 μmol) per kg body weight (equivalent to ~17 μmol phospholipid per mouse) diluted in 2 mL PBS was injected i.p. The large injection volume enabled widespread drug distribution in the peritoneal cavity, in a manner similar to that of clinical intraperitoneal therapy. The control group (i) was injected with 2 mL PBS.

2.2.1.3. LFUS treatment. One hour after drug injection, animals of all three groups were anesthetized using a ketamine–xylazine (85:15 by vol) solution (0.25 mL/g body weight) injected i.p. For LFUS treatment, the abdominal fur over the peritoneal cavity was removed using a hair-removal cream (Depicare, Careline, Yeruham, Israel), and a rubber cylinder (~16 mm in diameter), open at both ends, was sealed to the abdomen over the tumor, using a silicone paste (Baysilone, Bayer), and filled with water. A 20-kHz ultrasonic processor (VC400, Sonics & Materials, Newtown, CT) was used. The LFUS probe (13-mm diameter) was immersed in the water-filled cylinder, <2 mm above the skin. LFUS irradiation was conducted at an intensity of 5.9 W/cm^2 for 120 s at a continuous mode. Ultrasound intensity was determined calorimetrically [43], and reflects the energetic output normalized to the surface area of the tip.

2.2.1.4. Determination of liposome encapsulated and released cisplatin. About 3 h after drug injection, animals were sacrificed by ether inhalation. Then an additional 2 mL of PBS was injected into the peritoneal cavity, and the abdominal area was massaged to increase recovery of ascitic fluid and to free tumor cells. The ascitic fluid was aspirated with a syringe, and centrifuged (2000 rpm, 10 min) to separate cells from extracellular fluid. The extracellular fluid was chromatographed by gel permeation chromatography (GPC) to separate released cisplatin from liposomes (see Section 2.3.1). Cisplatin in the GPC fractions and in tumor cells was quantified by atomic absorption specroscopy (AAS) (Section 2.3.2). The phospholipid (PL) content of each fraction was extracted and quantified (Section 2.3.3).

2.2.2. Release of cisplatin in C26 tumors in the footpad

2.2.2.1. Tumor model. C26 murine colon adenocarcinoma cell medium consisted of 90 vol.% RPMI-1640 medium; 1 vol.% 0.2 mM

L-glutamine; 8.9 vol.% fetal calf serum (virus-screened); and 0.1 vol.% antibiotics (10 mg/mL streptomycin with 10,000 units/mL penicillin), all from Biological Industries, Beit Haemek, Israel.

Cells were grown under 5% CO_2 at 37 °C in 15-mL flasks and prepared for injection by first decanting the medium, and then adding 5 mL of Puck's saline solution (Biological Industries) containing trypsin (0.25%) and EDTA (0.05%) in order to free adsorbed cells. Cell medium (5 mL) was added to the trypsin–EDTA cell dispersion (2 mL), and the mixture was centrifuged (2000 RPM, 5 min, 25 °C). After removal of the supernatant, 2 mL PBS was added to the cell pellet. Cell concentration was determined under a microscope using a cell counting chamber (Neubauer, Marienfeld, Germany) and corrected with PBS to achieve a final concentration of ~10^6 cells per 50 µL.

Approximately 10^6 cells were injected subcutaneously into a rear footpad of 8-week-old BALB/c female mice (Harlan Laboratories, Ltd., Jerusalem, Israel). One week after cell inoculation, tumors were observed, and animals were divided randomly into six test groups (8 mice per group): (i) control, saline (placebo) plus LFUS; (ii) control, no drug and no LFUS; (iii) Stealth cisplatin without LFUS; (iv) Stealth cisplatin plus LFUS; (v) free (non-liposomal) cisplatin plus LFUS; (vi) free cisplatin without LFUS. The experiment was repeated twice.

Animals were weighed (±0.1 g) and tumors were sized (±0.01 mm) once a week.

2.2.2.2. Drug administration. All animals, treated with either free (non-liposomal) cisplatin or with liposomal cisplatin, received 5 mg cisplatin/kg body weight administered i.v. Drug administrations were given one and three weeks after cell inoculation. This drug dose correlates to 5.7 µmol phospholipid per mouse administered to mice treated with the liposomal drug.

Free cisplatin (Pharmachemie, Haarlem, Holland) was supplied in sterile injection vials of 1 mg cisplatin/mL.

2.2.2.3. LFUS exposure. 24 h after drug injection, in order to enable liposome accumulation at the target site, LFUS-treated animals were anesthetized using a ketamine–xylazine solution injected i.p. The foot with the tumor was immersed in a water bath (24 °C) and the LFUS probe was placed in the bath <2 mm from the skin. LFUS irradiation was conducted at an intensity of 5.9 W/cm^2 for 60 s at a continuous mode. A shorter LFUS exposure time was chosen, in comparison to the treatment time for the treatment of the abdominal tumor, due to the skin in this location being delicate.

2.2.3. Evaluating the effect of LFUS exposure on plasma levels of cisplatin

C26 tumors were developed in the footpad of BALB/c mice as described above. Animals were divided randomly into four groups (6 mice per group): (i) Stealth cisplatin without LFUS; (ii) Stealth cisplatin plus LFUS; (iii) free (non-liposomal) cisplatin plus LFUS; (iv) free cisplatin without LFUS.

2.2.3.1. Drug treatment. All animals in the drug treated groups received a single dose of 2.5 mg cisplatin per kg body weight, injected i.v.

24 h after drug injection animals in LFUS groups were treated as described above (see Section 2.2.2). Immediately after LFUS exposure, while animals were still anesthetized, blood was collected into K-EDTA-containing tubes by eye enucleation, followed by animal sacrifice by cervical dislocation. Blood samples from animals not exposed to LFUS were collected at the same time as blood samples from animals exposed to LFUS. Cisplatin level in blood was determined by AAS (see Section 2.3.2).

2.2.4. Evaluating the effect of LFUS on release of nSSL from the tumor

C26 tumors were grown in the footpad of BALB/c mice as described above (see Section 2.2.2). Animals were divided randomly into two groups (6 mice per group): (i) ^{3}H(CE)-radiolabeled nSSL with LFUS and (ii) ^{3}H(CE)-radiolabeled nSSL without LFUS.

2.2.4.1. Drug treatment. All animals received the same dose of 50 µL of the ^{3}H(CE)-radiolabeled nSSL injected i.v. 24 h after nSSL injection, and animals in the LFUS group were exposed to LFUS as described above (see Section 2.2.2).

2.2.4.2. Extracting ^{3}H(CE)-labeled liposomes from plasma and tissue. Before or immediately after LFUS irradiation, blood was collected, mice were sacrificed, and tumors were surgically removed. Tumors and blood were prepared for radioactivity counting using a tissue-dissolving agent (Solvable, Perkin Elmer, Waltham, MA), followed by immersion in a liquid scintillation cocktail (Ultima Gold, Perkin Elmer,), as described in the manufacturer's instructions [44].

Radioactivity level was determined using a β liquid scintillation counter (Betamatic, Kontron, Finland) and normalized to tissue weight.

2.3. Analytical procedures

2.3.1. Determination of in vivo cisplatin release from nSSL

Tumor extracellular fluid (200 µL) was chromatographed by GPC to separate free cisplatin from liposomes [17]. Columns were made of 10 mL plastic pipettes packed with ~1 cm of glass wool at their narrow bottom end (to prevent gel leakage) and loaded with Sepharose 6B (Sigma). The mobile phase consisted of 15 mM HEPES in 5% dextrose and physiological saline (9:1 v/v), pH 7.0. One milliliter fractions were collected, using a fraction collector (Amersham Biosciences), and quantified for their cisplatin content by AAS (see Section 2.3.2) and phospholipid content by the modified Bartlett assay [45], as described below (Section 2.3.3).

2.3.2. Cisplatin quantification

Levels of cisplatin remaining in the liposomes were quantified by Pt AAS, at 2700 °C (λ = 265.9 nm), using a Zeeman atomic absorption spectrometer SpectAA300 (Varian), with a sensitivity of ±1 ng Pt/mL (corresponding to ~±1.5 ng cisplatin/mL). A standard reference Pt solution (BDH Chemicals, Poole, UK) was used for calibration.

2.3.3. Phospholipid extraction and quantification

Lipids of the GPC fractions were separated by extraction from proteins, cisplatin and other water-soluble substances using the Bligh and Dyer extraction procedure [45]. The chloroform-rich lower phase (LP), which contained practically all the lipids, was separated from the methanol/water-rich upper phase (UP) by centrifugation for 5 min at 10,000 rpm, and washed with a synthetic chloroform/methanol/water (96:94:6 by vol) to remove traces of water-soluble compounds. The phospholipids in the chloroform-rich washed LP were then quantified by the modified Bartlett assay [45]. Although partition of cisplatin and its solubility in the LP is minimal [46], Pt remnants in the LP may still interfere with the modified Bartlett assay. Therefore, phospholipid determination was confirmed using the Stewart assay [47], in which Pt does not interfere.

3. Results and discussion

3.1. LFUS release of liposomal cisplatin in a J6456 murine lymphoma model and tumor cellular level of cisplatin

Stealth cisplatin was injected intraperitonealy to J6456-lymphoma-bearing mice. The abdominal area was then exposed to LFUS for 120 s. After exposure, tumor extracellular fluid and cells were aspirated and separated. Extracellular fluid was fractionated by GPC to separate liposomal from non-liposomal drug (only liposomes were found in the void volume). Drug levels in GPC fractions of extracellular fluid were determined by Pt analysis using AAS, and phospholipid levels were determined by the modified Bartlett assay (Fig. 1) [45]. Cisplatin found in the GPC void volume was considered to be liposome encapsulated.

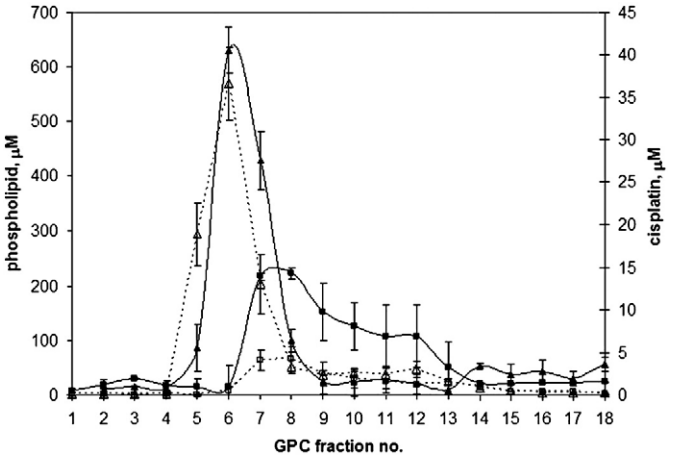

Fig. 1. Levels of cisplatin (dashed lines and hollow bars, *right axis*) and of liposomal phospholipids (solid lines and full bars, *left axis*) in GPC fractions of the lymphatic extracellular fluid of LFUS- (5.9 W/cm^2, 120 s) exposed (■, □), and non-exposed animals (▲, Δ) were compared. Levels of cisplatin were quantified by AAS, and of phospholipids by the modified Bartlett assay [45] (for details see Methods).

Measuring the drug-to-phospholipid mole ratio in liposomal fractions (Fig. 1), and comparing it to the drug-to-phospholipid mole ratio of the original Stealth cisplatin prior to injection (0.0581 mol cisplatin per mol phospholipid) enabled us to determine the amount of cisplatin released in vivo from the liposomes following exposure to LFUS. The drug-to-phospholipid mole ratio in liposome fractions of LFUS-exposed mice was 0.0176 (±0.0025, ±standard deviation), in comparison to 0.0565 (±0.0007) in animals not exposed to LFUS. These data show that LFUS applied in vivo induces a release of 69.6% (±4.4) of cisplatin from the liposomes, in comparison to almost no release (2.8%±1.1) in mice not exposed to LFUS, an ~25-fold increase in release due to exposure to LFUS. Irradiated liposomes in vivo were eluted at GPC fractions in agreement with our previous in vitro study on LFUS-irradiated liposomal cisplatin [36], and with other data regarding non-irradiated liposomal cisplatin in vivo [17], thus suggesting that most of the liposomes in vivo remain intact after LFUS irradiation. A summary of the drug release data appears in Table 1.

Quantifying the total phospholipid content in all *liposomal* GPC fractions shows a recovery of 74.6% (986±138 nmol PL/column) and indicates that LFUS irradiation reduced the liposomal phospholipid content by 25.4%, in comparison to non-irradiated mice, where almost no loss (>98% recovery) of liposomal phospholipids occurred (1323±116 nmol PL/column). Liposome phospholipid reduction after LFUS irradiation is similar to what we [36] (and others [48]) observed in vitro, in which disassembly of ~23% of the liposomes by LFUS, to form smaller phospholipid aggregates occurred [36]. The disassembly of some of the liposomes by LFUS is supported by the presence of a tail of phospholipids in GPC fractions of LFUS-exposed mice (Fig. 1), in comparison to the GPC phospholipid distribution in mice not exposed to LFUS (in which the phospholipid content is only in the void volume).

The content of cisplatin in tumor cells of LFUS-exposed mice, quantified by AAS of Pt, was 2.7-fold higher than in animals not exposed to LFUS (1666±276 and 616±164 ng/g cell, respectively). The high cellular cisplatin content in LFUS-treated animals seems to be due to enhanced cisplatin bioavailability following its release from the liposomes. Once cisplatin is released, it can diffuse and be taken up by tumor cells, where it is expected to bind to the nuclear DNA and exert anticancer activity [49]. However, the contribution of LFUS-induced enhanced cellular permeability of tumor cells, which has been shown in previous studies [33–35,50], cannot be ruled out.

As LFUS-induced cisplatin *release* was 25-fold higher than cisplatin release without LFUS, we expected a higher than 2.7-fold increase in cellular cisplatin level of LFUS-exposed animals [51]. This discrepancy requires further investigation.

3.2. Plasma levels of drug and lipids following LFUS irradiation

The effect of exposure to LFUS on the level of cisplatin in plasma was tested before and immediately after LFUS irradiation. Animals with a C26 tumor in a footpad were injected i.v. with either liposomal or free (non-liposomal) cisplatin. Twenty-four hours later (enabling liposomes to passively target the tumor [14,52]) the tumor was exposed to LFUS. In animals treated with liposomal cisplatin the levels of cisplatin in the plasma increased after LFUS irradiation from 24±4 (before LFUS) to 183±85 ng cisplatin/mL plasma after LFUS irradiation. These levels correlate to ~0.06% and ~0.4% of the total injected cisplatin dose, before and after LFUS irradiation, respectively. The maximal murine tolerated dose of cisplatin is ~9 mg cisplatin/kg body weight [53–55], which correlates with a plasma concentration of ~6000 ng cisplatin/mL (calculated from [56] and [57]). Namely, the measured level of cisplatin in plasma after LFUS exposure is ~3% of this value. The

Table 1
Effect of LFUS on cisplatin release from nano sterically stabilized liposomes — summary of major results.

Intraperitoneal J6456 murine lymphoma tumor		
	With LFUS	W/O LFUS
Percent of cisplatin released from liposomes	69.6±4.4%	2.8±1.1%
Tumor cell content of cisplatin	1666±276 ng cisplatin/g cell	616±164 ng cisplatin/g cell
Footpad C26 tumor		
	Before LFUS	After LFUS
Plasma levels of cisplatin in animals treated with liposomal cisplatin	24±4 ng cisplatin/mL plasma	183±85 ng cisplatin/mL plasma
Levels of ^{3}H-labeled liposome lipids in the tumor	13833±3132 CPM/g tumor	10578±2163 CPM/g tumor
Levels of ^{3}H-labeled liposome lipids in the plasma	71.8±26.3 CPM/mL plasma	96.2±19.6 CPM/mL plasma

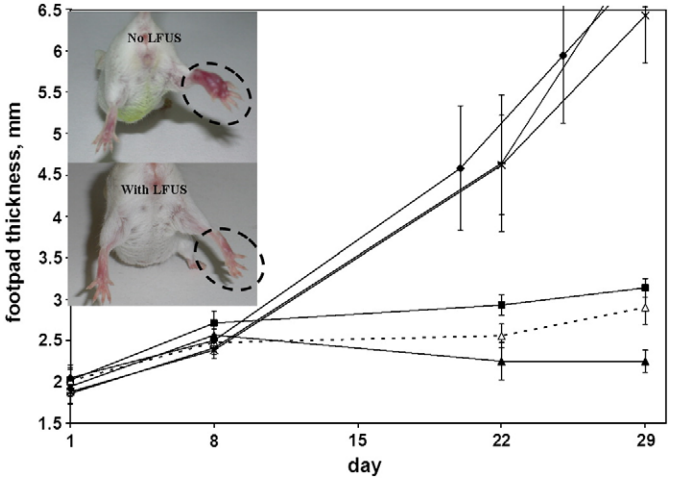

Fig. 2. C26 tumors in the footpad of BALB/c mice were exposed to different treatments: (i) control, no drug no LFUS (upper image in graph) (♦); (ii) control, saline (placebo) plus LFUS (5.9 W/cm^2, 60 s, -); (iii) nSSL cisplatin injected intravenously without LFUS (×); (iv) nSSL cisplatin injected intravenously plus LFUS (upper image in graph) (▲); (v) free (non-liposomal) cisplatin plus LFUS (–Δ–); (vi) free cisplatin without LFUS (■). Insert — footpad of mice treated with nSSL cisplatin i.v., without (top) or with (bottom) LFUS. At day 29, using Student's *t*-test, a statistically significant difference ($p<0.05$) was demonstrated between animals in group (iv) and group (v), and ($p<0.006$) between group (iv) and all other groups. Data points indicate mean footpad thickness of six mice, in two experiments, ±SD.

elevation of cisplatin level in plasma after irradiation may be due to the fact that liposomes reside in the tumor near blood vessels [14], thereby enabling permeation of small amounts of released cisplatin into circulation. Plasma levels of cisplatin in animals treated with free cisplatin before and after LFUS irradiation remained low (<20 ng cisplatin/mL plasma). These data are another indication of the ability to release cisplatin from liposomes in vivo using LFUS.

To test whether the elevation in plasma level of cisplatin was accompanied by the release of liposomes from the tumor matrix, animals with C26 tumors in the footpad were injected i.v. with liposomes labeled with the non-transferable non-metabolizable ^{3}H cholesteryl hexadecyl ether. Twenty-four hours later the tumors were exposed to LFUS. It was found that the levels of ^{3}H in the tumor and in plasma, before and after LFUS irradiation, did not change significantly ($13{,}833 \pm 3132$ and $10{,}578 \pm 2163$ CPM/g tumor, before and after LFUS irradiation, respectively, and 71.8 ± 26.3 and 96.2 ± 19.6 CPM/mL plasma, before and after LFUS irradiation, respectively), thereby indicating that although some of the drug is released to the circulation, LFUS exposure of the tumor does not release the vesicles (i.e., the liposomes) from the tumor site.

3.3. LFUS-induced tumor regression in C26 tumors in the footpad

The ability to affect the therapeutic efficacy by LFUS-induced localized drug release was assessed on BALB/c mice bearing a C26 tumor in a footpad (see Fig. 2 insert). All animals in the control groups, which included untreated mice, or mice exposed to LFUS and not treated with liposomal cisplatin, or mice treated with liposomal cisplatin without LFUS, had similar, fast-growing tumors (Fig. 2). These data indicate that neither exposure to LFUS alone, nor treatment with liposomal cisplatin alone, affects tumor proliferation.

The best therapeutic efficacy was achieved when mice treated with liposomal cisplatin were then exposed to LFUS (Fig. 2). These data indicate that localized release of a drug at the tumor site is therapeutically beneficial, most likely due to enhanced bioavailability of the drug at the tumor site. This treatment was superior to the treatment with free cisplatin followed by an exposure to LFUS, and even more so than free cisplatin alone (Fig. 2). The improvement in the group treated by free cisplatin combined with LFUS, in comparison to free cisplatin alone, may be due to increased cell/tumor permeability and drug uptake induced by LFUS [32,33,35,58]. These data confirm previously published in vitro data [36] showing that the chemical integrity and biological potency of LFUS-released drugs is retained after irradiation.

In Doxil (Caelyx™, in Europe), the anti-cancer drug doxorubicin is loaded into liposomes having an identical lipid composition and structure as the liposomes used in this study to encapsulate cisplatin. Myhr and Moan [39] tested the synergistic effect of treating mice bearing a tumor in the footpad with Doxil, administered intraperitoneally, and then exposing the tumor to LFUS for 30 min. Animals treated with a dose of 3 mg liposomal doxorubicin/kg body weight and exposed 1 h later to LFUS had smaller tumors than tumors treated with Doxil alone [39]. However, animals treated with a higher dose of Doxil (6 mg/kg BW) had similar tumors in the LFUS-treated and untreated groups [39], suggesting that the release level of doxorubicin from Doxil, even without LFUS, was sufficient to achieve therapeutic efficiency. These data suggest that LFUS should be applied primarily in cases that the spontaneous drug release level at the tumor site is therapeutically insufficient (as in the case of cisplatin), while in cases that the drug releases from the liposomes at a therapeutically effective rate (as in the case of Doxil) the use of LFUS may not provide any added value.

Other researchers, compared the release of doxorubicin from Doxil and from thermo-sensitive liposomes (ThermoDox™, composed of DPPC:MPPC:DSPE-PEG-2000 in a molar ratio of 90:10:4, respectively [59]), using high intensity focused ultrasound (HIFU) [28]. They found, in vitro, that using HIFU to elevate the temperature to 42 °C did not result in doxorubicin release from Doxil but did release doxorubicin from ThermoDox [28]. Similarly, combining Doxil with HIFU, in vivo, did not result in an improvement in the therapeutic efficacy, while combining Thermodox with HIFU did improve the therapeutic efficacy [28]. The lack of release of doxorubicin from Doxil by local heating is explained by the high mol% of cholesterol (~40 mol%) in its lipid bilayer. At these cholesterol levels, the whole lipid bilayer is transformed to the liquid-ordered phase [60], thus being highly rigid and exhibiting extremely low permeability and temperature sensitivity [61,62].

4. Conclusions

This study shows the ability to release a drug locally in vivo from nano sterically stabilized liposomes (nSSL) using low frequency ultrasound (LFUS). It was demonstrated that a short exposure to LFUS effectively releases the anti-cancer chemotherapeutic agent cisplatin from nSSL, thereby improving the therapeutic efficacy significantly. The local release of the drug in the tumor resulted in an elevation in plasma drug concentration, most likely due to intravasation of the released drug from the tumor to circulation. This study on liposomal cisplatin is an indication of the ability to apply LFUS for triggering the release of liposomal drugs having low in vivo release rates, and points to its potential for increasing the therapeutic efficacy of such liposomal formulations. As LFUS is difficult to focus, and dissipates rather near the body's surface, the applicability of LFUS is mainly for superficial tumors, as in the case of skin, and some head and neck and gynecological cancers. For deeper tumors, HIFU, and HIFU-responsive drug delivery systems, may be more suitable.

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