



Ultrasound triggered release of cisplatin from liposomes in murine tumors

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article info

Article history:

Received 4 December 2008

Accepted 3 March 2009

Available online 19 March 2009

Keywords:

Liposome

Low frequency ultrasound

Cisplatin

Cancer

Passive targeting

C26 adenocarcinoma

J6456 lymphoma

Murine tumor model

Controlled release

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.

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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/cm² 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) b 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 (m²⁰⁰⁰PEG 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 ³H-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, b 2 mm above the skin. LFUS irradiation was conducted at an intensity of 5.9 W/cm² 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 spectroscopy (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

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