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Efficient tumor regression by a single and low dose treatment with a novel and enhanced formulation of thermosensitive liposomal doxorubicin

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ABSTRACT

We have developed a novel and simplified thermosensitive liposomal formulation (HaT: *H*yperthermia-*a*ctivated cyto*T*oxic) composed of DPPC lipid and Brij78 (96:4, molar ratio). The HaT nanoparticles were loaded with doxorubicin (DOX) with >95% efficiency when a pH gradient method and a drug/lipid ratio of 1/20 (w/w) were applied. Drug release from the HaT formulation was significantly faster at 40–41 °C (100% release in 2–3 min) with 3.4-fold increased membrane permeability compared to the LTSL (lyso-lipid temperature sensitive liposomes; DPPC: MSPC: DSPE-PEG_{2000} = 86:10:4, molar ratio), a formulation that is currently in clinical trials. Both formulations displayed similar stability at 37 °C in serum (10–20% release in 30 min), which corresponds to their comparable pharmacokinetics in the unheated mice. An approximately 1.4-fold increased drug delivery to the locally heated tumor (~43 °C) was detected with HaT–DOX compared to LTSL–DOX. Moreover, when compared with free DOX, HaT enhanced drug uptake in the heated tumor by 5.2-fold and reduced drug delivery to the heart by 15-fold. A single i.v. treatment with HaT–DOX at 3 mg DOX/kg in combination with localized hyperthermia demonstrated enhanced tumor regression compared to LTSL–DOX and free DOX, and exhibited little toxicity.

1. Introduction

Nanoparticle (NP)-based drug delivery systems including liposomes enable targeting of anticancer drugs to tumors, and their development and optimization have been a major focus in the field of drug delivery. Encapsulating anticancer drugs in liposomes has been demonstrated to improve the therapeutic window by enhancing antitumor efficacy and reducing side effects [1]. Furthermore, the blood circulation time of liposomes can be prolonged by modifying the particles with polyethylene glycol (PEG), which acts to reduce the uptake of the carriers by the mononuclear phagocyte system (MPS) [2]. The prolonged circulation time of the liposomes increases their accumulation in solid tumors by the enhanced permeability and retention (EPR) effect [3], wherein the liposomes enter the tumor via a leaky vasculature, and are not easily eliminated due to the compromised lymphatic system. PEGylated liposomal doxorubicin (DOX) (Doxil®/Caelyx®) has been approved clinically for Kaposi sarcoma, multiple myeloma and advanced ovarian cancer. However, while Doxil® has minimized the acute cardiotoxicity associated with free DOX, it does not substantially increase the efficacy compared to free DOX in the clinical setting. It is now widely understood that release of DOX from Doxil® is slow (<5% in 24 h), leading to limited bioavailability (40–50%) [1,4–6]. Additionally, NPs accumulating in tumors are concentrated around the microvessels with limited tumor penetration and exposure [7,8]. To overcome the release and penetration issues, attention in the drug delivery field has focused on designing NPs capable of releasing a drug when exposed to a specific triggering mechanism [8,9]. Triggers include decreased pH in the tumor microenvironment, light, ultrasound, enzymatic action or heat (reviewed in [10]).

Among the trigger-sensitive NP formulations that have been developed, thermosensitive liposomes are the most advanced in commercial and clinical development, as focused induction of hyperthermia in deep tissue is clinically feasible and permissible [11]. Precise and localized heating can be achieved by application of technologies including imaging-guided radiofrequency ablation [12], focused microwave, and focused ultrasound [13–15]. Studies have demonstrated that the use of hyperthermia in combination with the thermosensitive liposomal delivery results in increased intratumoral free drug concentrations compared to levels achieved with free drug or liposomal drug administered in the absence of heating, leading to significantly enhanced antitumor efficacy [13,16–22]. The most clinically advanced thermosensitive liposomal formulation is composed of DPPC/MSPC/DSPE-PEG_{2000} (86/10/4, molar ratio), described as lyso-lipid temperature sensitive liposomes (LTSL) [11,13,16,17,23–25] and is currently in Phase III clinical trials for liver cancer and Phase II for recurrent breast cancer on chest wall (www.celsion.com). When heated to 42 °C, the LTSL released its full DOX content rapidly [13,23,24,26,27], leading to a ~15-fold increase in drug delivery to the heated tumor [16] and eradication of the *s.c.* inoculated human xenograft tumor in a mouse model when given at 5 mg DOX/kg [16,17].

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The LTSL–hyperthermia approach is differentiated from conventional liposomal therapy by a non-dependence on the EPR effect. The thermosensitive liposomes are administered during the hyperthermia treatment, with immediate release of the encapsulated drug in the vasculature of the heated tumor [28]. The liposomes have the effect of maintaining a high blood concentration of a drug by reducing renal clearance: as the liposomes circulate within a hyperthermic tumor, the drug is released within a finite volume, generating a high drug concentration gradient, and driving diffusion of the drug from the blood into the tumor. In other words, the LTSL approach does not require accumulation of the liposomes in the tumor space via extravasation through leaky blood vessels, a process which is less efficient and highly tumor model dependent [29]. Furthermore, the LTSL/hyperthermia strategy is particularly advantageous for the delivery of toxic drugs to localized and inoperable tumors, for which there are currently limited and therapeutically disappointing treatment options [11]. Finally, this approach requires an ultrafast releasing mechanism, as blood passage time through the heated tumor is short. In addition, drugs with high cell membrane permeability are optimal, in order to drive fast cellular uptake and minimize rapid wash out from the tumor.

The objective of the study is to develop a more effective and simplified liposomal formulation for drug delivery under mild hyperthermia (40–42 °C). We focused on a liposomal formulation comprising a minority component of Brij78 (polyoxyethylene (~20 units, MW~880) stearyl ether, 4 mol%) and a majority component of DPPC lipid. Brij78 is a non-ionic surfactant consisting of a PEGylated acyl chain: it was hypothesized that Brij78 could replace the functions of MSPC, a lyso phospholipid (surfactant), and DSPE-PEG_{2000}, a PEGylated lipid which reduces opsonization and improves pharmacokinetics (PK). Accordingly we formulated Brij78 containing liposomes, quantified the drug release from the liposomes in mild hyperthermia conditions, and compared the drug delivery efficiency to locally heated tumors (43 °C) with the free drug and LTSL formulations.

2. Materials and methods

2.1. Materials

1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), 1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphatidylcholine (MSPC) and 1,2-distearoyl-*sn*-glycero-3-phosphatiylethanol-amine-N-[methoxy (polyethyleneglycol)-2000] (DSPE-PEG_{2000}) were purchased from Avanti Polar Lipids (Alabaster, AL). Brij78 [polyoxyethylene (20) stearyl ether] was obtained from Sigma Aldrich (Oakville, ON). DOX was purchased from Tocris Bioscience (Ellisville, MO). All other reagents were of analytical grade.

2.2. Preparation of DOX-loaded liposomes

The liposomes were prepared by the thin film hydration method, followed by membrane extrusion for size control as described previously [24]. Briefly, 15 mg of lipids were dissolved in isopropanol (IPA) and dried at 65 °C under a stream of nitrogen gas, and the resulting thin lipid film was placed under high vacuum for at least 2 h to remove residual organic solvent. The lipid film was hydrated with 1 ml 300 mM citric acid to form multilamellar vesicles (MLVs). The MLVs were sonicated for 10 min and subsequently extruded 10 times through stacked polycarbonate filters of 0.1 μm pore size at 65 °C to adjust liposome size, and the formulation was then cooled to room temperature.

The loading of DOX into liposomes were performed as described previously [24] with minor modifications. The exterior buffer of the liposomes was replaced by HBS (25 mM HEPES Buffered Saline, pH 7.4) via dialysis (Slide-A-Lyzer 10 kDa MWCO, Pierce Biotechnology, Rockford, IL) for 3 h against three exchanges of 500× volumes of HBS. The liposome suspension (10–15 mg/ml) and DOX were mixed at 1:20 (w/w, drug/lipid), and the mixture was incubated at 37 °C for 90 min. After incubation, un-encapsulated DOX was removed by gel filtration on a Sepharose CL-4B column (Sigma-Aldrich, St Louis, MO) equilibrated with HBS. The eluted liposome fraction was analyzed for lipid and drug content. The liposomal phospholipid concentration was quantified by the Fiske and Subbarow phosphate assay [30]. The DOX concentration was determined by measuring the fluorescence after the disruption of liposomes with Triton X-100 (10 μl of 1% Triton X-100 for 200 μl of diluted liposomes, final DOX conc. = 1–3 μg/ml, no heating required), using a Chameleon multi-label plate reader (Hidex Personal Life Science, Hidex Oy, Finland) (Ex 485 nm/Em 590 nm): signals were converted into concentrations units by comparing against a standard curve. The particle size and zeta potential (in 1.5 mM NaCl, 5% dextrose) of the liposomes were determined with a particle analyzer (Zetasizer Nano-ZS, Malvern Instruments Ltd, Malvern, UK). The encapsulation efficiency was calculated as [DOX/lipid (after gel filtration)]/[DOX/lipid (before gel filtration)] × 100%. The mean diameters of the DPPC/Brij78 and LTSL formulations were measured to be 90–110 nm with a PDI < 0.1. Encapsulation efficiency of DOX was >95%. For the following content, the DPPC/Brij78 (96/4, molar ratio) formulation is referred to as HaT (*H*yperthermia-*a*ctivated cyto*T*oxic) and LTSL is comprised of DPPC/MSPC/DSPE-PEG_{2000} = 86/10/4 (molar ratio). Incorporation of 4 mol% Brij78 was shown to be optimal for thermosensitive drug release in preliminary testing. All the following experiments were performed with freshly prepared formulations.

2.3. Differential scanning calorimetry (DSC) analysis and membrane permeability of liposomes

DSC analyses were performed to analyze the phase transition of LTSL and HaT formulations using the method reported earlier [31]. Membrane permeability rate constant of HaT and LTSL liposomes at 37, 40 and 42 °C was determined using the nitrobenzoxadiazole (NBD) quenching method as described previously [24].

2.4. In vitro release of DOX from liposomes

Measurement of DOX released from the liposomes was demonstrated as described previously [24]. The liposomes (1 μg DOX/ml in 200 μl of 50% FBS/50% HBS) were incubated at different temperatures (37–42 °C) for various time points and were immediately put on ice and transferred into a 96-well plate. The release of DOX was determined using a Chameleon plate reader by measuring the fluorescence (Ex 485 nm/Em 590 nm). The percentage of the released DOX was calculated as $(I_T - I_0)/(I_{100} - I_0) \times 100\%$, in which I_T is the fluorescence at time point t, I_0 is the fluorescence at the start of the incubation time, I_{100} is the fluorescence after the addition of 10 μl of 1% Triton X-100. The drug release rate constants at different temperatures were obtained by fitting the release curves with the first order kinetics (Fraction of drug release $(F) = 1 - e^{-kt}$, k = release rate constant, t = time) as described in the literature [32].

2.5. Animals and tumor cells

Female BALB/c mice (aged 5–6 weeks, 18–20 g) were purchased from The Jackson Laboratory (Bar Harbor, ME). All experimental protocols in this study were approved by the Animal Care Committee of the University Health Network (Toronto, Ontario, Canada) in accordance with the policies established in the Guide to the Care and Use of Experimental Animals prepared by the Canadian Council of Animal Care.

The mouse mammary carcinoma cell line EMT-6 was a generous gift from Dr. David Stojdl at the CHEO Research Institute and Dr. Douglas Mahoney at the University of Ottawa. EMT-6 cells were maintained in DMEM supplemented with 10% heat-inactivated fetal