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Optimization of a novel and improved thermosensitive liposome formulated with DPPC and a Brij surfactant using a robust in vitro system

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ABSTRACT

The combination of thermosensitive liposomes and local heating has been shown to improve anticancer drug delivery in both animal models and human patients. The lyso-lipid temperature sensitive liposomes (LTSL) consisting of DPPC, MSPC and DSPE-PEG_{2000} is currently under evaluation in clinical trials. We hypothesized that Brij surfactants resembling the chemical structures of MSPC and DSPE-PEG_{2000} could be utilized for generating a thermosensitive formulation with DPPC. Here, we report using a robust in vitro system to efficiently screen a series of liposomal candidates composed of DPPC and a Brij surfactant for thermosensitive delivery of doxorubicin. The data indicated that the optimal acyl chain length of the surfactant was between C_{16} and C_{18} with a saturated carbon chain, a PEG repeating unit ranging between 10 and 100 and a molecule weight above 600 Da. The linking chemistry between the acyl chain and the PEG chain did not influence thermosensitivity. In the panel of surfactants tested, Brij78 was optimal and could be incorporated into the liposomes by the thin film hydration or the post-insertion method with an optimal range of 1 to 8 mol%. Doxorubicin was incorporated into the formulation by pH gradient with >95% loading efficiency at drug/lipid of 1/20 (w/w). The transition temperature of the Brij78-liposomes was slightly lower than that of LTSL (41 v.s. 41.5 °C), leading to enhanced drug release at the low end of the hyperthermic temperatures (40 °C) with similar stability at 37 °C, which was confirmed by cell based assays. Finally, the Brij78-liposomes and LTSL displayed comparable blood compatibility with mild hemolytic activity. This in vitro system allowed for efficient screening and optimization to produce an optimal formulation.

1. Introduction

A major obstacle in current anticancer chemotherapy is low tumor selectivity due to the non-targeted delivery of the drug, producing considerable adverse effects. Nascent tumor vasculature is leaky and malignant tissue lacks lymphatic drainage: as a result of these two physiological features, nanoparticle (NP) drug delivery vehicles (including liposomes) can selectively accumulate in the tumor compartment, a phenomenon termed as the enhanced permeability and retention (EPR) effect [1]. If drug release from the NP occurs within the tumor, enhanced efficacy and reduced systemic side effects have been observed [2]. PEGylated liposomal doxorubicin (Doxil®/Caelyx®) exhibits prolonged circulation time and enhanced accumulation into tumor tissue, and minimizes the acute cardiotoxicity associated with free doxorubicin (DOX). The Doxil® formulation is the first approved nanomedicine for cancer therapy and is clinically indicated for Kaposi sarcoma, multiple myeloma and advanced ovarian cancer. However, the Doxil® formulation does not substantially increase the chemotherapeutic efficacy of DOX in the clinical setting. It is now believed that the compromised activity of Doxil®, despite its increased tumor targeting, is largely due to poor drug release from the nanoparticles (<5% in 24 h), leading to limited bioavailability [2–5]. Similar low bioavailability has been encountered with liposomal cisplatin (CDDP) (SPI-077), which demonstrates substantial tumor accumulation but displays no antitumor activity in clinical trials [6]. Again, the release of the membrane impermeable CDDP was confirmed to be minimal from the liposomal formulation [7]. Therefore a novel strategy to boost targeted drug release from nanoparticles to tumors is needed.

The use of mild hyperthermia (39–43 °C) to improve drug delivery to tumors has been demonstrated as a clinically feasible approach [8–11]. First, mild hyperthermia increases tumor perfusion and improves drug uptake, and also renders tumor cells temporarily sensitive to other treatments [11]. Additionally, hyperthermia can be applied locally to a tumor by image-guided radiofrequency [12] or focused ultrasound [13–15], permitting a mechanism for triggering localized drug delivery. Encapsulating a drug into thermosensitive liposomes can reduce the renal excretion and metabolism or uptake of the drug in other compartments and focus the dose to a locally heated target: the drug can be efficiently released within the vasculature of the heated tumor, generating a high drug concentration gradient and driving diffusion of the drug to the tumor cells. This combination of local heating and

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thermosensitive liposomes results in increased intratumoral free drug concentrations and significantly enhanced antitumor efficacy [10,13,16–20], and this concept is being evaluated in Phase III clinical trials for hepatocellular carcinoma and Phase II for recurrent breast cancer on chest wall (www.celsion.com). This thermosensitive liposomal formulation (LTSL: lyso-lipid temperature sensitive liposomes or ThermoDOX®) contains three lipid components: DPPC/MSPC/DSPE-PEG_{2000} = 86/10/4 (molar ratio) [10,13,16–20] and can be triggered to rapidly release DOX at elevated temperatures (40–43 °C).

Brij® molecules are PEGylated single-chain surfactants, commercially available with different PEG chain lengths and acyl chain structures. It was hypothesized that Brij surfactants could replace the functions of MSPC, a single acyl chain lipid/surfactant, and DSPE-PEG_{2000}, a PEGylated lipid/surfactant in the LTSL formulation, to prepare a simplified thermosensitive liposome. Indeed, the HaT formulation (Hyperthermia-activated cytoToxic) composed of DPPC and Brij78 exhibited enhanced drug release rates at 40–41 °C with equivalent blood pharmacokinetics in the mice compared to LTSL, leading to 1.4-fold increased drug uptake in the locally heated tumor and enhanced tumor regression after a single dose at 3 mg DOX/kg [21]. With these encouraging results, it was reasonable to assume that other Brij molecules might produce similar or even better formulations with DPPC compared to HaT. In this study, we investigated liposomes formulated with DPPC and a series of Brij surfactants, and identified structures or features required for triggering thermosensitive drug release using an efficient in vitro system. This information will be beneficial for rational design of a further improved formulation. Optimization on the formulation is also reported with this robust screening system. The optimal formulation was compared with LTSL in cell based assays, confirming its superiority in delivering doxorubicin under hyperthermia. The blood compatibility of the formulations was also tested.

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). Brij® and Myrj® surfactants were obtained from Sigma Aldrich (Oakville, ON). DOX was purchased from Tocris Bioscience (Ellisville, MO). All other reagents were of analytical grade. 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% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C with 5% CO_2.

2.2. Liposome preparation

The DOX-loaded liposomes were prepared using a method described previously with some minor modifications [19]. Three types of liposomal formulations were prepared: 1. Brij-liposomes (DPPC/Brij = 96/4, molar ratio), 2. Myrj-liposomes (DPPC/Myrj = 96/4, molar ratio) and 3. lyso-lipid temperature sensitive liposomes (LTSL, DPPC/MSPC/DSPE-PEG_{2000} = 86/10/4, molar ratio) (positive control). Fifteen milligrams of lipids mixed with surfactant were dissolved in isopropanol (IPA) and dried at 65 °C under a gentle 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 thin lipid film was hydrated with 300 mM citric acid (pH 2) to obtain a 20 mM liposome suspension. After sonication and membrane extrusion (at 65 °C, 100 nm membrane) to control the size, the liposomes were cooled to room temperature. The exterior buffer of the liposome suspension 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 at room temperature. The liposome suspension and DOX were mixed at 1:20 (w/w, drug/lipid), and the mixture was incubated at 37 °C for 90 min [19,22,23]. 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 as described previously [19,24]. The particle size and zeta potential of the liposomes were determined by 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%. HaT formulation (Hyperthermia-activated cytoToxic) is defined by the DPPC/Brij78 = 96/4 (molar ratio) composition and the thin layer chromatography analysis was performed to confirm no lysolipid generation due to hydrolysis of DPPC [21]. Alternatively, HaT formulation was prepared using the post-insertion method [25]. After the preparation of DPPC liposomes (~110 nm, PDI < 0.1) with the pH gradient, DOX was loaded using the method described above. The liposomes were then gently mixed with an aliquot of Brij78 solution (1, 2, 4 or 8 mol%, prepared in HBS), and incubated at 37 °C for 1 h. The complete insertion of Brij78 onto the liposomes was confirmed by the disappearance of the micelle peak of Brij78 (10 nm) determined by the Zetasizer Nano-ZS. The formulation was then purified on a Sepharose CL 4B column to remove free DOX and Brij78, if any.

2.3. Differential scanning calorimetry (DSC) analysis

DSC analyses were performed to analyze the phase transition of the liposomal formulations using the method reported earlier [19].

2.4. In vitro release of DOX from liposomes

Measurement of DOX released from the liposomes in HBS was demonstrated as described previously [23].

2.5. Cellular uptake and cytotoxicity of different liposomes activated by hyperthermia

The method of the cellular uptake and cytotoxicity study was adapted from that described previously [23] to verify drug release in serum containing culture media after a short exposure of hyperthermia (40 and 42 °C, 3 min). EMT-6 cells were seeded at a density of 2.5×10^4 cells/well in 500 μl culture medium in a 24-well plate. One day later, the medium was replaced with the liposomes that had been diluted with culture medium to a concentration of 30 μM DOX and heated at 37, 40, or 42 °C for 3 min. The cells were incubated for 4 h at 37 °C, after which the cells were gently washed two times with chilled PBS, and lysed by the addition of 0.5 ml of 0.3% Triton-X/PBS solution with agitation on a rotating platform for 15 min at room temperature. Acidified IPA (75 mM HCl, 10% water/90% IPA) (1.5 ml/well) was added to the lysate and the mixture was incubated at 4 °C in the dark for overnight. The cell lysate was collected and centrifuged for 3 min at 12,000 × *g*, and the supernatant was analyzed for the fluorescence intensity using a plate reader (Ex 485 nm/Em 590 nm). Cellular internalized DOX was completely extracted by this method as verified by no residual fluorescence on the culture plate detected by fluorescence microscopy. The protein content of the lysate was measured with the protein assay kit (Bio-Rad Laboratories, Hercules, CA), based on the Bradford method [26]. The data of intracellular uptake of DOX are expressed as fluorescence/mg protein. Intracellular uptake of DOX (30 μM) was also observed by fluorescence microscopy (Axio Observer Z1, Carl Zeiss, Göttingen, Germany) with the Axiovision software (Carl Zeiss) after multiple wash and fixation of the cells with 1% formalin in PBS for 15 min at room temperature.