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Optimized thermosensitive liposomes for selective doxorubicin delivery: Formulation development, quality analysis and bioactivity proof

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ABSTRACT

In our study we examined thermosensitive liposomal formulations (TL) from the perspective to minimize the general toxicity drawbacks of chemotherapy. The TL become active in response to local hyperthermia (LH), and remain inactive at physiological conditions. Here, we formulated novel doxorubicin loaded thermoliposomes (Dox-TL) with optimized characteristics and tested their biological activity *in vitro*. The liposomal membrane composition of Dox-TL and their preparation technology were adjusted for high drug loading and extended formulation stability. The 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC):1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC):cholesterol(Chol):1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG-2000) in molar ratio 9:1:0.2:0.02:0.2 and drug/lipid weight ratio 0.13–0.20/1 composition has demonstrated best results. The freshly-prepared vesicles contained 94% doxorubicin. The Dox-TL, freeze-dried with 4% sucrose, maintained high level of encapsulated drug, remained stable in serum and prevented premature drug leakage. The Dox-TL proved to be significantly less toxic at 37 °C than free Dox. In combination with local hyperthermia of 42.5 °C Dox-TL were as effective as free Dox in cell survival, and even outperformed free Dox in proliferation activity suppression, colony proliferation rate, and cellular uptake. These findings represent a solid basis for a safer and more effective antitumor therapy.

1. Introduction

Doxorubicin (Dox) is a well known anthracycline antibiotic with highly expressed antitumor activity that is broadly applied in modern clinical practice. Having a wide range of advantages, the Dox's bottleneck is its poor selectivity, which may cause a large number of side effects [1–3]. The cardiotoxicity is very common, and being life threatening for patients, causes serious drawbacks in cancer treatment with Dox. The therapeutic selectivity and better antitumor efficacy of Dox can be improved by encapsulation in liposomes. In modern cancer therapy several liposomal formulations have already been introduced (e.g. Doxil®) and are available on the world market [4–6]. These liposomal formulations contain PEG, which protects the liposomes from being captured by a phagocytic guard system. This so called "stealth" effect of PEG-modified liposomal membranes allows a higher concentration of Dox in blood for a longer period of time [7–10]. The enhanced permeability and retentive nature of tumor tissue enables the better targeting of tumors [11], whereas healthy organs will be avoided by any "inertly packed" active agent.

The thermoliposomes (TL) combined with local hyperthermia (LH) treatment can also increase the selectivity and efficacy of Dox [12]. The thermosensitive carriers respond with the release of cytostatic agents at the elevated temperature of 40–43 °C at the tumor site [13–15]. During LH the liposomal membrane, which normally prevents the drug-tissue contact, develops porosities and releases the encapsulated Dox [16]. The new drug formulation ThermoDox® (Celsion Corporation and Duke University, USA) which is used in combination with high frequency ablation, has launched the phase III of clinical trails for hepatocellular cancer. Despite being well investigated, only several liposomal formulations with thermal trigger could reach the late clinical phases and be launched to the world market [17,18].

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With the present study we address the optimization of thermosensitive liposomal doxorubicin formulation (Dox-TL), which will reduce the previous shortcomings of this drug carrier type, such as poor stability, poor selectivity and reproducibility [19,20]. The here-presented preparation technology improves drug loading, prevents drug leakage and increases colloidal stability. Selection of an appropriate cryoprotectant during the freeze-drying procedure makes the formulation appropriate for storage with minimal drug loss, which is essential for a longer shelf life and better availability for patients. The developed freeze-dried Dox-TL has been tested in cell culture in combination with LH to verify its efficacy and toxicity.

2. Materials and methods

2.1. Materials

All lipids: 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) (Lipoid GmbH, Germany), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG-2000) and cholesterol were purchased from Avanti Polar Lipids, Inc., USA. Polycarbonate membrane was obtained from Nuclepore, Whatman, United Kingdom and nylon membranes from Pall Corporation, San Diego, USA. Doxorubicin was purchased from OAO ONOPB Moscow, Russia. Other chemicals were purchased from AppliChem GmbH, Darmstadt, Germany. All chemicals and solvent were of analytical grade commercially available.

2.2. Preparation of thermoliposomes

To prepare empty TL the method of phase reversion was applied [21]. All lipids were dissolved in chloroform, after solvent evaporation under vacuum the lipid film was obtained. Under constant stirring and warming up to 50 °C the lipid film was hydrated with 250 mM Ammonium sulfate solution. The obtained dispersion of multilayer vesicles was extruded through polycarbonate membrane with 100 and 200 nm pore size at 50 °C using the Avanti Mini-Extruder (Avanti Polar Lipids, Inc., USA), 21 consequent cycles were made.

Doxorubicin (Dox) was loaded into TL using ammonium sulfate gradient [22], the process was performed with 20-fold dilution in HEPES buffer containing cryoprotectant (4% sucrose) at pH 8.4. The freshly-prepared dispersion of Dox-TL was filtered using nylon membranes with the pore size of 0.45 μm and then sterile filtered through 0.22 μm membrane. For stabilization the obtained Dox-TL were freeze-dried. The formulation purification from ammonium sulfate was done with Sephadex® column as described in Section 2.4 for Dox.

2.3. Size measurement, morphology and stability

2.3.1. Dynamic light scattering

The vesicle hydrodynamic diameter and size distribution were analyzed by dynamic light scattering (Nicomp 380 Submicron Particle Sizer, Particle Sizing Systems, USA). The liposomal dispersion was diluted in 1:100 phosphate buffered saline (PBS) to the concentration applicable for measurement and analyzed in triplicates using 5-mW HeNe laser (632.8 nm) at 90° scattering angle. The liquid viscosity was set at 0.933 mPa s as for water at 23 °C, index of refraction of the solvent was set 1.333 (refraction of water). To optimize the efficiency of the autocorrelation process the average scattered intensity was set to 300 kHz. The obtained results were presented as an average value of at least 3 replicates with standard deviation.

2.3.2. Cryofracture scanning electron microscopy (CryoSEM)

The CryoSEM images were obtained using a JSM-7500F (Jeol, Tokyo, Japan). The ALTO-2500 liquid nitrogen (LN2) cryotransfer system (Gatan Inc., Pleasanton, CA, USA) enabled sample microscopy at −140 °C. Liposome suspension drop were shock frozen between special holders and transferred in nitrogen cooled system, where the freeze-fracture was performed by knocking off one of the holders. Fracture surface was sputtered with a thin platinum layer directly using the ALTO system. Carriers were examined at an accelerating voltage of 4 kV.

2.3.3. Dox-TL stability in serum

The stability of Dox-TL was tested in the presence of γ-irradiated fetal calf serum (FCS) as reported previously [23]. In short, the 20 μL of liposomal suspension were added to 180 μL of FCS already adjusted to 25 or 37 °C. The 2% Triton-X was applied as a 100% release control. After incubation for 1 h and dilution 1:50 with Tris/NaCl the Dox-TL were measured at the Ex. λ = 485/Em. λ = 580 nm for Dox release.

For the monitoring of possible changes in hydrodynamic diameter of thermoliposomes in FCS, they were measured at different time points at 25 and 37 °C in triplicates.

2.4. Purification of liposomes via column chromatography (gel filtration)

The Dox-TL dispersion was purified from not encapsulated drug using gel filtration on chromatographic column. For quantification of drug encapsulation efficacy the drug content in the dispersion was evaluated. One millilitre of Dox-TL dispersion was applied on the Sephadex® filled chromatographic column C10/20 G-50 Superfine (Amersham Biosciences, Sweden). 0.15 M solution of NaCl was used as eluent at the elution speed of 0.3–0.4 mL/min. The purification process was controlled via UVis-920 detector and REC 111 recorder (Amersham Biosciences, Sweeden), monitoring the peak separation.

2.5. Quantitative analysis of Dox in Dox-TL

The Dox content in Dox-TL was measured with spectrophotometry using the standard sample (RSO) Dox at the wavelength of 252 ± 2 nm, and calculated in mg as follows Eq. (1):

$$X = \frac{D \cdot a \cdot C}{D_o \cdot C_o} \quad (1)$$

where D – is the sample optical density, D_o – the optical density of (RSO) Dox, C – dilution rate of the sample, C_o – dilution rate of the (RSO) Dox, a – weight of (RSO) Dox in mg.

The encapsulation efficacy of Dox in Dox-TL (B, %) was calculated as follows Eq. (2):

$$B = \frac{D_1 \cdot C_1 \cdot V_1}{D \cdot C \cdot V} \times 100 \quad (2)$$

where D_1 – the optical density of the solution fraction of purified Dox-TL, D – the optical density of solution of initial Dox-TL dispersion; C_1 – dilution rate of the fraction dilution with purified Dox-TL; C – dilution rate of the fraction of initial Dox-TL dispersion; V_1 – fraction volume of the purified Dox-TL in mL; V – fraction volume of the initial Dox-TL dispersion applied on the column in mL.

2.6. In vitro biological activity

2.6.1. Cell cultivation

The B16-F10 mouse melanoma was cultivated in RPMI-1640 culture medium supplemented with fetal calf serum (FCS) 10% at