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A comparison study between lycobetaine-loaded nanoemulsion and liposome using nRGD as therapeutic adjuvant for lung cancer therapy

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ABSTRACT

To achieve tumor-selective drug delivery, various nanocarriers have been explored using either passive or active targeting strategies. Despite the great number of studies published annually in the field, only nanocarriers using approved excipients reach the clinical stage. In our study, two classic nanoscale formulations, nanoemulsion (NE) and liposome (Lipo) were selected for the encapsulation of lycobetaine (LBT). To improve the lipid solubility of LBT, oleic acid (OA) was used to complex (LBT-OA) with lycobetaine (LBT). Besides, PEGylated lecithin was used to enhance the circulation time. The release behaviors of LBT from non-PEGylated and PEGylated NE and Lipo were compared. PEGylated LBT-OA loaded Lipo (LBT-OA-PEG-Lipo) exhibited a sustained release rate pattern, and *in vivo* pharmacokinetic profiles showed the extended circulation compared nanoemlusions. Besides, LBT-OA-PEG-Lipo showed an enhanced anti-tumor effect in the mice xenograft lung carcinoma model. Moreover, a multi-target peptide nRGD was co-administered as a therapeutic adjuvant with LBT-OA loaded formulations, which demonstrated improved tumor penetration and enhanced extravasation of formulations. Also, co-administration of nRGD significantly improved the *in vivo* antitumor efficacy of different formulations, likely due to the depletion of tumor-associated macrophages (TAMs). Thus, LBT-OA-PEG-Lipo + nRGD may represent a promising strategy for cancer chemotherapy against lung carcinoma.

1. Introduction

Lung cancer is one of the most commonly diagnosed malignancies with high morbidity and mortality globally (Siegel et al., 2014). Despite advances in chemotherapeutics to improve survival, the median survival remains limited to < 12 months (Ferlay et al., 2015). Lycobetaine (LBT), a quaternary phenanthridinium alkaloid from *Lycoris radiate* of the Amaryllidaceae family (Lee et al., 1994), has been proven cytotoxic on Lewis lung carcinoma (Ghosal et al., 1988; Lu et al., 2013), nasopharyngeal epidermoid carcinoma (Wang et al., 1987) and 21 other human tumor xenografts (Barthelmes et al., 2001) by inhibiting topoisomerases I and II (Casu et al., 2011; Xu, 1991). However, the half-life of LBT in blood was about 30 s, which greatly limited its application in clinic (Zhao et al., 2013).

Over the years, great advances have been made in nanotechnology. Nanocarriers are proven useful to achieve targeted delivery, extend half-life, and reduce the systemic toxicity in chemotherapy. Marketed nanotherapeutic products include nanocrystals, liposomes, nanoemulsions, nanoparticles and polymer-protein conjugates (Hafner et al., 2014). Among various nanocarriers with or without targeting ligands, nanoemulsions (NEs) and liposomes (Lipos) consisting of lecithin, oil or cholesterol are the most popular nanoformulations in clinic mainly due to the safety of excipients used in these formulations (Hosokawa et al., 2002a; Hosokawa et al., 2002b; Sarbolouki et al., 2000; Sun et al., 2011). Oli-in-water nanoemulsions are suitable templates for drug delivery due to high encapsulation efficiency, easy water-dispensability, sufficient physical stability and high bioavailability (McClements and Rao, 2011). Our group previously reported that PEGylated LBT-oleic acid nanoemulsion (LBT-OA-PEG-NE) underwent rapid metabolism and elimination within 10 h in rats, indicating a relatively short circulation time *in vivo* (Zhao et al., 2013). Prolonged circulation time was thus preferred in nanoscale carriers, which would lead to an enhanced accumulation at the tumor site resulting from the enhanced permeability and retention (EPR) effect (Maeda, 2010; Wang et al., 2013). Besides NEs, liposomes are extensively studied for encapsulating both hydrophilic and hydrophobic chemotherapeutics (Hafner et al., 2014). Liposome consists of one or more lipid bilayers enclosing an aqueous core. This structure allows the incorporation and delivery the lipo- or hydrophilic molecules. Besides, the liposome surface can be modified by polymers, peptides, or antibodies (Allen and Cullis, 2004; Hafner

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et al., 2014). PEGylated liposomes have been widely used in clinic, for example, Doxil®, the first liposome formulations launched in the early 1990s, was used for treatment of ovarian and recurrent breast cancers (Svenson, 2012). However, only few nanoemulsions have been commercialized due to limitations related to usage of surfactants or cosolvents, and issues of drug precipitation (Narang et al., 2007).

To seek a suitable carrier system for LBT, both NE and liposomes were selected for encapsulation of LBT, and comparative studies were performed to elucidate the release behaviors of LBT from formulations including LBT-OA-NE, LBT-OA-PEG-NE, LBT-OA-Lipo and LBT-OA-PEG-Lipo.

Our group previously reported a multi-target peptide nRGD consisting of alanine-alanine-asparagine (AAN) and cyclic iRGD as a highly efficient targeting ligand with significantly improved anti-tumor efficacy of doxorubicin-loaded liposomes *in vivo* (Xu Song et al., 2016). With the iRGD module, nRGD was hypothesized to interact with α_v integrin receptors and neuropilin-1 (NRP-1) to mediate tumor target and penetration, while the AAN module may selectively be cleaved by overexpressed legumain in the tumor tissue thus achieving tumor-specific drug delivery (Xu Song et al., 2016). In the previous study of iRGD, Sugahara et al. found when iRGD used as an adjuvant, it also contributed to the increased vascular and tissue permeability without drug modification (Sugahara et al., 2010). Therefore, in this study, nRGD was also used as a therapeutic adjuvant for tumor penetrability and TAMs-depletion.

Thus, we aimed to compare the *in vitro* and *in vivo* antitumor efficacy of two LBT-OA loaded nanocarrier systems, *i.e.* NE *vs.* Lipos, and to elucidate the impact of PEGylation on the *in vivo* profiles of these systems. Most important of all, we coadministrated LBT-OA loaded NEs/Lipos with nRGD, and would like to elucidate the impact of physically mixed nRGD on the antitumor efficacy of LBT-OA loaded formulations. Specifically, the encapsulation efficiency, X-ray photoelectron spectroscopy (XPS), release study, and bioavailability of LBT-OA loaded NE (LBT-OA-NE) and PEGylated NE (LBT-OA-PEG-NE), LBT-OA loaded Lipo (LBT-OA-Lipo) and PEGylated Lipo (LBT-OA-PEG-Lipo) were investigated systematically.

2. Materials and methods

2.1. Materials, cell culture and animals

Lycobetaine (LBT) acetate was obtained by Sichuan Shifang Hongsheng Plant Raw Material Co, Ltd. (Shifang, Sichuan, China). nRGD peptide (AAN-iRGD, CRGDK(NAA)GPDC) with a C-terminal cysteine was synthesized (purity 95%) by GL Biochem (Shanghai, China). Purified yolk lecithin (Lipoid E80) and oleic acid was purchased from Lipoid Co. Ltd. (Ludwigshafen, Germany). Cholesterol was obtained from Chengdu Kelong Chemical Company (Chengdu, Sichuan, China). Soybean oil was offered by Sichuan Baili Pharmaceutical Group Co, Ltd. (Chengdu, Sichuan, China). MPEG2000-DSPE was purchased from were purchased from Advanced Vehicle Technology (Shanghai, China). 1, 10-dioctadecyl-3, 3, 30, 30-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonatesalt (DiD) was purchased from Biotium (USA). 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Beyotime Institute Biotechnology (Haimen, China).

Lewis lung carcinoma cell line (LLC), human A549 non-small cell lung cancer cell line and murine melanoma cells (B16) were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle's Medium (high glucose) (GIBCO Products International, Langley, OK, USA) supplemented with 10% fetal bovine serum (Minhai Bio-Engineering Co, Ltd., Gansu, China), 100 U/mL streptomycin and 100 U/mL penicillin (Hyclone, Logan city, USA) at 37 °C in a 5% CO_2 humidified environment incubator.

Male Sprague-Dawley rats (200 ± 20 g), male C57/BL6 mice (20 ± 2 g) were purchased by the Experimental Animal Center of Sichuan University. The animal experiment protocols and procedures were performed according to the requirements of the National Institutes of Health Policy and National Act under the approval and supervision of the Animal Ethics Committee in Sichuan University.

2.2. Preparation of LBT-OA

LBT-OA was prepared as the previous report (Zhao et al., 2013). Briefly, a certain amount of sodium bicarbonate solution was added into the aqueous solution of LBT acetate to form the aqueous solution of LBT base, and LBT-OA was prepared by adding ethanol solution of OA (25 mg) into the LBT base solution (containing 10 mg of LBT). After vortexed, a cloudy solution was spontaneously formed due to the complex formation, and the LBT-OA, a yellow precipitate, was collected by centrifugation and freeze drying.

2.3. Preparation of LBT-OA loaded NE and LBT-OA loaded Lipo

LBT-OA loaded NE was prepared as previous report (Zhao et al., 2013). Briefly, LBT-OA prepared from 10 mg LBT and 25 mg OA, 80 mg lipoid E80 and 50 μL soybean oil were dissolved in 20 mL of dichloromethane and the organic phase was removed by rotary evaporation (Buchi Laboratory Equipment, Flawil, Swizerland) at 45 °C to form a lipid film. The film was gently hydrated with 10 mL of deionized water at 37 °C. After sufficient hydration, the pre-dispersion was passed through a high-pressure homogenizer (EmulsiFlex-C5, AVESTIN Inc., ON, Canada) for seven cycles to form LBT-OA-NE. Besides, LBT-OA-PEG-NE was prepared at the same way by replacing 80 mg lipoid E80 with 76 mg lipoid E80 and 15 mg mPEG2000-DSPE (Table S1).

LBT-OA loaded liposome was prepared as the same way. LBT-OA prepared from 10 mg LBT and 25 mg OA, 80 mg lipoid E80 and 40 mg cholesterol were dissolved in dichloromethane and the organic solvent was removed by rotary evaporation at 45 °C until a thin lipid film formed. The obtained film was gently hydrated with 10 mL of deionized water at 37 °C, and then further passed through a high-pressure homogenizer for seven cycles to form LBT-OA-Lipo. Moreover, the PEGylated liposome was prepared at the same way by replacing 80 mg lipoid E80 with 76 mg lipoid E80 and 15 mg mPEG2000-DSPE (Table S1).

2.4. Characterization of NE and liposome

Particle size and zeta potential of NE and liposome were analyzed by photon correlation spectroscopy (Malvern Zetasizer Nano ZS90, Malvern Instruments, Malvern, UK) at 25 °C. Equilibration time was set at 120 s.

The morphologies of NE and liposome were observed with transmission electron microscopy apparatus (TEM, H-600, Hitachi, Japan). Samples were negatively stained with 2% phosphptungstic acid (PTA) (Gu et al., 2013a).

2.5. Determination of encapsulation efficiency (EE)

To determine the entrapment efficiency of different formulations, two different method were used, ultrafiltration (Yue et al., 2009) and sephadex filtration (Verma and Ahuja, 2015). Nanosep Centrifugal Filtration Devices (MWCO 30 kDa, Pall Corporation, Port Washington, NY, USA) were used to separate the free drug from the formulation. 400 μL of freshly prepared LBT-OA-NE, LBT-OA-PEG-NE, LBT-OA-Lipo, LBT-OA-PEG-Lipo (LBT 1 mg/mL) was added to the sample reservoir tube and centrifuged at 4000 rmp for 15 min. The collected filtrate was diluted with 1% acetic acid in ethanol, and the origin formulation was diluted at the same way. Then the concentration of LBT was analyzed by fluorescence spectrophotometry RF5301 PC (Shimadzu Scientific Instruments, Kyoto, Japan) at the excitation wavelength 370 nm and the emission wavelength 552 nm. The EE of LBT in different