European Journal of Pharmaceutics and Biopharmaceutics 99 (2016) 73–83

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research Paper

Dual drug loaded nanoliposomal chemotherapy: A promising strategy for treatment of head and neck squamous cell carcinoma

Aarti Mohan [a], Shridhar Narayanan [b,1], Gopalan Balasubramanian [b], Swaminathan Sethuraman [a], Uma Maheswari Krishnan [a,*]

[a] Centre for Nanotechnology & Advanced Biomaterials, School of Chemical & Biotechnology, SASTRA University, Thanjavur 613 401, Tamil Nadu, India
[b] Orchid Chemicals and Pharmaceuticals Pvt. Ltd., Sozhinganallur, Chennai 600 119, Tamil Nadu, India

ARTICLE INFO

Article history:
Received 23 June 2015
Revised 27 November 2015
Accepted in revised form 29 November 2015
Available online 9 December 2015

Keywords:
Oral cancer
Liposomes
Resveratrol
Doxorubicin hydrochloride
Apoptosis

ABSTRACT

The rising incidence of head and neck cancer and the drawbacks of currently used therapeutic strategies such as salvage surgery followed by adjuvant chemo- or radiotherapy have encouraged pursuits for better therapeutic approaches. This work describes the development and characterization of a PEGylated liposomal nanocarrier encapsulated with *trans*-resveratrol (Res), a plant stilbenoid, and doxorubicin hydrochloride (Dox), a standard chemotherapeutic agent for treatment of oral squamous cell carcinoma. The two drugs were loaded in liposomes prepared from egg phosphatidylcholine and DSPE-PEG with maximum encapsulation efficiencies of about 80% for each drug achieved at Res to Dox ratio of 2:1. The liposomal suspension was found to be stable with a zeta potential of −30.53 mV and size of approximately 250 nm. Thermal properties and release kinetics of the dual drug loaded liposomes were determined. The nanoformulation was evaluated for its *in vitro* anticancer efficacy on an oral squamous cell carcinoma cell line (NT8e). The cell uptake mechanism of the liposomal formulation was determined using pharmacological inhibitors for different endocytosis pathways. The combination effect of the two drugs was evaluated in free form and was found to have synergistic effects. The formulation was found to have a higher IC_{50} value than that of free doxorubicin hydrochloride but was found to have a superior effect on the signaling proteins involved in apoptosis and cell cycle.

1. Introduction

Cancer therapy has remained a tough challenge for centuries. Surgery, either used alone or followed by chemo- or radiotherapy, has been the ideal therapeutic modality to treat solid tumors of different origins and stages [1]. However, these treatments are always accompanied by painful side effects thereby reducing the quality of life in cancer patients. The use of liposomes to efficiently deliver chemotherapeutic drugs to the tumor site has been explored by several researchers [2,3]. There are also commercially available liposomal nanoparticles (LipoPlatin, Marquibo® Doxil®, Myocet®, CAELYX™), which have been shown to exhibit longer circulation times and facilitate prolonged release of the drug thereby resulting in reduced toxicity.

Doxorubicin hydrochloride (Dox) is used widely to treat breast cancer, acute myelogenous leukemia (AML), acute lymphoblastic leukemia (ALL), gastric cancer, neuroblastoma, Hodgkins lymphoma, etc. However, its side effects include soreness of skin, irritation, alopecia, fatigue, etc. Though liposomal formulations of Dox namely Doxil®, Myocet® and CAELYX™ aid in targeting the drug to tumor cells and reduced its toxicity to a certain extent, the undesirable effects were not completely eliminated [4]. The drawbacks accompanied with traditional methods of therapy have led to investigation of safer alternatives such as plant polyphenols for chemotherapy. Among the several hundred plant compounds that have been examined for their anticancer properties, *trans*-resveratrol (Res) has emerged as a popular choice. Various groups have described its anticancer [5], anti-diabetic [6], cardioprotective [7], anti-inflammatory [8] and neuro-protective [9] effects in detail. However, the drawbacks associated with resveratrol are its sparse water solubility resulting in poor bioavailability and its derivatization on entering the physiological system [10,11] leading to a reduction in its therapeutic efficacy. In this context,

* Corresponding author at: Departments of Chemistry, Bioengineering & Pharmacy, Centre for Nanotechnology & Advanced Biomaterials (CeNTAB), School of Chemical & Biotechnology, SASTRA University, Thanjavur 613 401, Tamil Nadu, India. Tel.: +91 4362 264101x3677; fax: +91 4362 264120.
E-mail address: umakrishnan@sastra.edu (U.M. Krishnan).
[1] Formerly at Orchid Chemicals and Pharmaceuticals Pvt. Ltd., Sozhinganallur, Chennai 600 119, Tamil Nadu, India.

http://dx.doi.org/10.1016/j.ejpb.2015.11.017
0939-6411/

co-administration with another drug that could offset the negative aspects of resveratrol could serve to enhance its therapeutic potential.

Several groups have worked on studying the effect of using two or more anticancer drugs with supplements to produce enhanced efficacy combinations of chemotherapeutic drugs [12–14]. Doxorubicin has been used in combination with several phytochemicals to treat different cancers [15,16]. The combination of Dox and Res has been explored for treatment in breast cancer [17–20] and the cardio-protective effects of resveratrol (Res) have been studied in Dox induced cardiotoxicity models [21]. Studies are required to understand the molecular targets of this potent combination in other forms of cancer. One of the drawbacks in conventional multi-drug chemotherapeutic regimens is the possibility of the drugs not being available in the target cells at the same time. Encapsulation of the drugs involved in a single nanocarrier can overcome this hurdle. Though such strategies have not been reported for Dox and Res, the combination of Dox with another well-known phytochemical curcumin loaded into a core–shell polymeric nanoparticle has been investigated using pancreatic cancer cell lines and it was found to be successful in overcoming drug resistance [22]. The dual encapsulation of Dox with curcumin in micelles also has been found to inhibit lung cancer [23]. The present work therefore attempts for the first time to achieve dual encapsulation of these two drugs in a nano-dimensional liposome. The physical and chemical characterization of the nanoformulation and its anti-tumor activity in a head and neck squamous cell carcinoma cell line have also been described in this study.

2. Materials and methods

2.1. Materials

L-α-phosphatidylcholine (egg/chicken) (EPC) and Disteroylpho sphatidylethanolamine-poly ethylene glycol (2000) (DSPE-PEG) were purchased from Avanti Polar Lipids, USA, and Northern Lipids, USA respectively. *trans*-resveratrol (Res) and doxorubicin hydrochloride (Dox) were kind gifts from Orchid Chemicals and Pharmaceuticals, Chennai, India, and NATCO Pharmaceuticals, India, respectively. The salts for preparation of phosphate buffered saline, chloroform, methanol and DMSO were procured from Merck, India. The head and neck squamous cell carcinoma cell lines, NT8e [24] was provided by Dr. Rita Mulherkar formerly at Advanced Centre for Training, Research and Education on Cancer (ACTREC), Mumbai. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum, and penicillin/streptomycin were purchased from Gibco, Invitrogen, India. The antibodies, RIPA extraction buffer and protease inhibitor cocktail were purchased from Cell Signaling Technologies, USA, and Sigma Aldrich respectively. The enhanced chemiluminescence (ECL) detection kit was procured from Thermo, USA, and bovine serum albumin (BSA) was procured from SRL, India.

2.2. Methods

2.2.1. Preparation and characterization of drug-loaded liposomes

The drug-loaded liposomes were prepared by passive loading technique. A thin layer of lipids (EPC and DSPE-PEG) and Res dissolved in chloroform and methanol respectively, was made by evaporating the solvents in vacuum. Dox was dissolved in distilled water (pH 7) and added in the hydration medium. The liposomes were prepared by heating the lipid drug suspension to 60 °C with constant stirring for around 45 min. Subsequently the suspension was extruded through polycarbonate membrane of pore size 200 nm (Liposofast Basic, Avestin, Canada) at around 50 °C. The drug-loaded liposomes were centrifuged at 8453.25 g to separate free Res and at 28177.5 g to separate liposomes from free Dox.

2.2.2. Encapsulation efficiency

The drug-loaded liposomes were separated from free drug by centrifugation and the liposomal pellet was dissolved using methanol. Res and Dox were quantified by measuring the absorbance at 306 nm and 482 nm respectively using UV–visible spectrophotometer (Lambda 25, Perkin Elmer, USA) and the amount of drug was determined using a standard graph. The percentage encapsulation was calculated using the following equation:

$$\text{Encapsulation Efficiency}\ (\%) = \frac{\text{Amount of drug encapsulated}}{\text{Total drug}} \times 100$$

2.2.3. Thermal analysis

The drug-loaded liposomes were prepared as mentioned earlier and 5 mg was used to analyze its thermal properties using a differential scanning calorimeter (Q20, TA Instruments, USA). The sample was placed in an aluminum pan that also served as the reference and analyses were carried out at a scan rate of 2 °C per minute in an inert atmosphere of nitrogen.

2.2.4. Size and morphology

The blank and dual drug loaded liposomes were prepared as described before and the size, zeta potential and colloidal stability of liposomes were measured using the Zeta sizer (Malvern Instruments, UK). Colloidal stability was determined by incubating the liposomal suspension at 37 °C in phosphate buffered saline (pH 7.4) under gentle stirring conditions and aliquots were analyzed for particle size distribution at intervals of 6 h for a period of 24 h. The morphology of the blank and dual drug loaded formulations were analyzed using field emission transmission electron microscope (FE-TEM) (JEM 2100F, JEOL, Japan). The liposomal suspension was dried on a copper grid, stained with 1% phosphotungstic acid and imaged at 200 keV.

2.2.5. Release kinetics

Dialysis bags (Himedia, India) were immersed in boiling distilled water for over 1 h to remove any preservative and successively rinsed with PBS (pH 7.4). The drug-loaded liposomal samples were added to the dialysis bags, sealed at both ends and suspended in 200 μL release medium. The bag was then immersed completely in 25 mL release medium and gently stirred while maintaining the temperature at 37 °C. Release kinetics were studied in three different release media *viz.* phosphate buffered saline (PBS) (pH 7.4), simulated saliva solution (SS) (pH 6.8) and simulated saliva solution supplemented with 1% fetal bovine serum (SF). The composition of simulated saliva solution is 16.7 mM Na_2HPO_4, 1.39 mM KH_2PO_4 and 0.9% NaCl [25]. One milli liter aliquots were withdrawn at each time point and replaced with equal amount of fresh release media thus maintaining sink conditions. Both drugs were estimated using a standard graph for each and percentage release was calculated.

2.2.6. Cell uptake studies

The mechanism of cell uptake of dual drug-loaded liposomes was investigated using pharmacological inhibitors for three different endocytosis pathways namely caveolin-mediated endocytosis (genistein, 50 μM), clathrin-mediated endocytosis (chlorpromazine, 10 μM) and micropinocytosis (amiloride, 50 μM). NT8e cells were maintained in DMEM supplemented with 1% antibiotic and 10% FBS at 37 °C in a humidified 5% CO_2 incubator. The cells were trypsinized using 0.025% Trypsin–EDTA (Gibco, Invitrogen) every third day. NT8e cells were seeded and pretreated with these