Biomedicine & Pharmacotherapy 97 (2018) 489–495

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

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

Original article

Paclitaxel-loaded folate-coated long circulating and pH-sensitive liposomes as a potential drug delivery system: A biodistribution study

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ARTICLE INFO

Keywords:
Breast cancer
Scintigraphic images
MDA-MB-231 tumor
Paclitaxel
pH-sensitive liposomes
Folate

ABSTRACT

A range of antitumor agents for cancer treatment is available; however, they show low specificity, which often limit their use. Recently, we have reported the preparation of folate-coated long-circulating and pH-sensitive liposomes (SpHL-folate-PTX) loaded with paclitaxel (PTX), an effective drug for the treatment of solid tumors, including breast cancer. The purpose of this study was to prepare and characterize SpHL-PTX and SpHL-folate-PTX radiolabeled with technetium–99 m (^{99m}Tc). Biodistribution studies and scintigraphic images were performed after intravenous administration of ^{99m}Tc-PTX, ^{99m}Tc-SpHL-PTX and ^{99m}Tc-SpHL-folate-PTX into healthy and tumor-bearing mice. High radiochemical purity (> 98%) and *in vitro* stability (> 90%) were achieved for both liposome formulations. The pharmacokinetic properties of ^{99m}Tc-SpHL-DTPA-PTX and ^{99m}Tc-SpHL-folate-DTPA-PTX decreased in a monophasic manner showing half-life of 400.1 and 541.8 min, respectively. Scintigraphic images and biodistribution studies showed a significant uptake in liver, spleen and kidneys, demonstrating these routes as way for excretion. At 8 h post-injection, the liposomal tumor uptake was higher than ^{99m}Tc-PTX. Interesting, 4 h after administration, the liposome folate coated showed higher tumor-to-muscle ratio than ^{99m}Tc-SpHL-DTPA-PTX and ^{99m}Tc-PTX. In conclusion, the liposomal systems, showed high tumor uptake by scintigraphic images, especially the ^{99m}Tc-SpHL-folate-DTPA-PTX that showed a sustained and higher tumor-to-muscle ratio than non-functionalized liposome, which indicate its feasibility as a PTX delivery system to folate positive tumors.

1. Introduction

Cancer is a public health worldwide problem, due to its high prevalence and mortality. A range of antineoplastic agents is available for clinical applications; however, their low specificity leads to toxic effects in healthy tissues, which, in many cases, could limit their use [1–5]. Among the chemotherapeutic agents, paclitaxel (PTX) is one of the most effective and potent drugs used in the treatment of several solid tumors including, breast, ovarian, non-small cell lung cancer, head and neck tumors [3,6–8]. PTX is poorly soluble in water, therefore, it is commercially available as a micellar dispersion in Cremophor EL® (polyethoxylated castor oil) and dehydrated ethanol (1:1 v/v), which allows its administration by the intravenous route. Nevertheless, several drawbacks have been related to their clinical application, such as hypersensitivity reactions, peripheral sensory neuropathy, and myelosuppression, besides development of drug resistance [3,6,9–12]. To overcome these problems, liposomes have emerged as an interesting platform for effective drug delivery since they are biocompatible, biodegradable and nontoxic nanosystems. In addition, encapsulation into liposomes might prevent drug degradation, improve drug solubility and reduce drug distribution to undesired tissues [3,6,13,14]. Nowadays, some formulations with PTX into different nanosystems have been reported and approved for clinical applications. Among them, a conventional liposomal preparation made up of phospatidylcholine and phosphatidylglycerol (in a 9:1 molar ratio, respectively) containing PTX, Lipusu®, was approved in China, in 2006 for the treatment of ovarian, breast, head and neck cancer, gastric and non-small cell lung carcinoma [3,6,15–17]. In South Korea, a polymeric micelle

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http://dx.doi.org/10.1016/j.biopha.2017.10.135
Received 4 September 2017; Received in revised form 18 October 2017; Accepted 21 October 2017

formulation, Genexol-PM®, another PTX-nanosystem, has been approved for breast cancer treatment, and, in 2005 FDA approved the Abraxane®, a PTX albumin-bound nanoparticle formulation, for the treatment of metastatic cancer [18–22]. In general, these nanosystems demonstrated *in vivo* efficacy similar to Taxol®, however some studies have demonstrated their similar toxicity as well low selectivity to tumor tissue, after intravenous injection [11,15–22]. In other to overcome this drawback, we reported the preparation of PTX-loaded folate-coated long circulating and pH-sensitive liposomes (SpHL-folate-PTX). The liposomal formulation is composed by dioleylphosphatidylethanolamine (DOPE), cholesteryl hemisuccinate (CHEMS), distearoylphosphatidylethanolamine-polyethylene glycol$_{2000}$ (DSPE-PEG$_{2000}$) and distearoylphosphatidyl-ethanolaminepolyethyleneglycol$_{2000}$-folate (DSPE-PEG$_{2000}$-folate) in the molar ratio of 5.7:3.8:0.45:0.05, respectively [3]. Previous studies using small angle X-ray diffraction clearly demonstrated the pH-sensitive of SpHL-PTX showing that the presence of CHEMS led to the stabilization of DOPE molecules in a lamellar structure at pH 7.4. Nonetheless, at lower pH, *i.e.* at tumor tissues, CHEMS is protonated leading to a hexagonal phase, which is essential to release the drug from liposomes [14]. SpHL-PTX revealed a high level of PTX leakage when in contact with an acid medium. Significant difference in PTX leakage was obtained from pH 6.8, and a release around 30% higher were observed. At pH 5.0 a release next to 70.0% were achieved which confirms the pH-sensitivity of the system. *In vitro* studies on MDA-MB-231 cells, a human breast tumor line, showed a higher cytotoxic activity for liposomal formulation in comparison to the free drug. Worth mentioning was the improved cytotoxicity of folate-coated formulation which suggests a higher uptake of the vesicles explained by superexpression of folate receptors in this cell line [3]. Due to these promising results, it is essential to evaluate the biodistribution profile of these nanoparticles in order to determine the real potential of these formulations as PTX delivery systems. As such, the purpose of this study was to prepare and characterize SpHL-PTX and SpHL-folate-PTX, radiolabeled with technetium–99 m (^{99m}Tc). Biodistribution studies and scintigraphic images were performed after intravenous administration of ^{99m}Tc-PTX, ^{99m}Tc-SpHL-PTX and ^{99m}Tc-SpHL-folate-PTX into healthy and tumor-bearing mice.

2. Materials and methods

2.1. Materials

Paclitaxel was supplied by Quiral Quimica do Brasil S.A (Juiz de Fora, Brazil). Cremophor EL® and $SnCl_2{\cdot}2H_2O$ were purchased from Sigma-Aldrich (São Paulo, Brazil). Dioleoylphosphatidylethanolamine (DOPE) and distearoylphosphatidyl-ethanolaminepolyethyleneglycol$_{2000}$ (DSPE-PEG$_{2000}$) were acquired from Lipoid GmbH (Ludwigshafen, Germany). Cholesteryl hemisuccinate (CHEMS) was supplied from Sigma Chemical Company (St. Louis, USA). Sodium chloride (NaCl) was obtained from Merck (Rio de Janeiro, Brazil). Acetonitrile HPLC grade was purchased from Fischer Scientific (New Jersey, USA). ^{99m}Tc was obtained from an alumina-based ^{99}Mo/^{99m}Tc generator. Water was purified using a Milli-Q apparatus (Millipore, Billerica, USA). All other chemicals and reagents used in this study were of analytical grade. MDA-MB-231 (human breast adenocarcinoma) cell line was purchased from American Type Culture Collection (ATCC® HTB-26™) (Manassas, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, penicillin and streptomycin were supplied by Gibco Life Technologies (Carlsbad, USA). Trypsin-EDTA solution (0.5%) and trypan blue were purchased from Sigma-Aldrich (São Paulo, Brazil). Matrigel was acquired from BD Biosciences (Bedford, MA). Female BALB/c mice (6–8-week-old) were obtained from CEBIO-UFMG (Belo Horizonte, Brazil) and BALB/c nude mice (6–8-week-old) were supplied from IPEN-SP (São Paulo, Brazil). All animal studies were approved by the local Ethics Committee for Animal Experiments (CEUA/UFMG) under the protocol number 409/2013.

2.2. Synthesis of distearoylphosphatidyl-ethanolaminepolyethyleneglycol$_{2000}$-Diethylene-triaminepentaacetic acid (DSPE-PEG$_{2000}$-DTPA)

The DSPE-PEG$_{2000}$-DTPA was synthetized as previously described [15]. Briefly, a solution of DSPE-PEG$_{2000}$-NH$_2$ in DMSO (40.0 mg/ml) was added to DTPA dianhydride in DMSO:pyridine 7:3 (v/v) (32.0 mg/ml). The mixture was heated in an oil bath under constant stirring for 90 min at 100 °C. Then, ultrapure water was added to the reaction and mixture was maintained at 100 °C, for 90 min. The solvent was evaporated and the product was re-suspended in water and purified by dialysis using a Spectrapore® membrane with a 1.0 kDa cut-off, at room temperature for 36 h. The final product was lyophilized in a 24 h cycle and stored at −20 °C.

2.3. Liposomes preparation

Liposomes composed of DOPE, CHEMS e DSPE-PEG$_{2000}$ and DSPE-PEG$_{2000}$-DTPA (SpHL-DTPA-PTX) at a molar ratio of 5.7:3.8:0.45:0.05, respectively, were prepared using the standard lipid film hydration method [3]. In brief, pre-determined chloroform aliquots of the lipids and PTX (0.5 mg/ml) were transferred to round bottom flask and a lipid film was obtained by evaporating the organic solvent under reduced pressure. Next, to promote the complete ionization of CHEMS molecules, an aliquot of NaOH solution (0.456 M) was added at a 1:1 molar ratio CHEMS:NaOH. The film was hydrated with NaCl 0.9% (w/v), followed by vigorous shaking in vortex. The vesicles were sonicated (20% amplitude) in an ice bath for 5 min using a high-intensity ultrasonic processor (R2D091109 model; Unique® Instruments, Indaiatuba, Brazil). The suspension were submitted to a centrifugation process (Sigma 4k-15 centrifuge, Sigma Laborzentrifugen GmbH, Osterode, Germany) at 3000 rpm at 4 °C for 10 min to eliminate non-entrapped PTX. Since the drug shown low water solubility, it precipitate and a drug pellet is formed. The supernatant suspension represent the final and purified formulation. For the folate-coated liposomes, 0.05% of DSPE-PEG$_{2000}$-folate was added to the lipid film formation.

2.4. Physicochemical characterization

2.4.1. Mean diameter and zeta potential

The mean diameter of SpHL-DTPA-PTX and SpHL-DTPA-folate-PTX was determined by dynamic light scattering (DLS) at 25 °C and at a fixed angle of 173°, using Zetasizer NanoZS90 (Malvern Instruments, England). The Zeta potential of the samples was measured by DLS associated to the electrophoretic mobility using the same equipment. All the samples were analyzed after 10-fold dilution in filtered NaCl 0.9% (w/v) solution (cellulose ester membrane, 0.45 mM, Millipore). Data were expressed as the mean ± standard deviation (SD) of at least three different batches.

2.4.2. Drug encapsulation percentage

The percentage of drug encapsulation of PTX in SpHL-DTPA-PTX or SpHL-DTPA-folate-PTX was determined by high performance liquid chromatography (HPLC) based on the determination of PTX concentration in the liposomes before (non-purified liposomes) and after centrifugation (purified liposomes) [24]. The liposomal vesicles were disrupted using isopropanol in a volume ratio of 1:10 and later diluted in a mixture of acetonitrile:water (55:45 v/v). This dispersion was filtered through a 0.45 μm Millex HV filter (Millipore, Billerica, MA, USA) and injected in the chromatographic apparatus. The encapsulation percentage (EP) was calculated using the following equation and the data were expressed as the mean ± standard deviation of at least three different liposomal formulation measurements:

$$EP = \frac{[PTX]\,purified\ \ liposomes}{[PTX]\,nonpurified\ \ liposomes} \times 100$$