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Experimental design of a liposomal lipid system: A potential strategy for paclitaxel-based breast cancer treatment

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ABSTRACT

Paclitaxel (PTX) is widely used as a first-line treatment for patients with metastatic breast cancer; however, its poor water solubility represents a major challenge for parenteral administration. The encapsulation of the PTX in drug-delivery systems with high affinity for tumor sites could improve the uptake and increase its therapeutic efficacy. In this work, long-circulating and pH-sensitive PEG-coated (SpHL-PTX) and PEG-folate-coated liposomes containing PTX (SpHL-FT-PTX) were prepared, and the physicochemical properties and in vitro cytotoxic activity were evaluated. Both formulations presented adequate physicochemical properties, including a mean diameter smaller than 200 nm, zeta potential values near the neutral range, and an encapsulation percentage higher than 93%. Moreover, SpHL-FT-PTX showed a good stability after storage for 100 days at 4 °C. The viability studies on breast cancer cell lines (MDA-MB-231 and MCF-7) demonstrated cytotoxic activity more pronounced for SpHL-FT-PTX than for SpHL-PTX or free drug for both tumor cell lines. This activity was reduced to a rate comparable to SpHL-PTX when the cells were previously treated with folic acid in order to saturate the receptors. In contrast, in the normal cell line (L929), cell viability was decreased only by free or liposomal PTX in the highest concentrations. A significantly higher selectivity index was obtained after SpHL-FT-PTX treatment compared to SpHL-PTX and free PTX. Therefore, the results of the present work suggest that SpHL-FT-PTX can be a promising formulation for the treatment of metastatic breast cancer.

1. Introduction

Although chemotherapy plays a central role in breast cancer treatment, the lack of specificity of conventional chemotherapeutic agents for tumor cells is one of the major problems in the therapy. Cytotoxic drugs affect both healthy and cancerous tissue; as a result, they often cause severe side effects, which limit their clinical application [1,2].

Paclitaxel (PTX) is one of the most effective and potent anticancer drugs used against a wide range of solid tumors, namely breast cancer, ovarian cancer, non-small cell lung cancer, head and neck tumors, Kaposi's sarcoma, and urologic malignancies [3,4]. PTX is poorly soluble in water and thus, until recently, the commercially available preparation, Taxol®, consisted of micellar dispersion of PTX in Cremophor EL® (polyethoxylated castor oil used as a solubilizing surfactant) and dehydrated ethanol (1:1 v/v). Cremophor EL® improves the PTX solubility and allows its administration by intravenous route. However, Taxol® therapy is associated with serious dose-limiting toxicities such as myelosuppression, peripheral sensory neuropathy, allergic reactions, and eventual development of drug resistance [4,5].

To overcome these PTX-related disadvantages, the development of PTX delivery systems, including the use of liposomes, polymeric nanoparticles, micellar dispersions, and cyclodextrin complexes, has been thoroughly investigated [6]. Liposomal formulations have been suggested as potentially better options for delivery of this drug

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since they can minimize detrimental toxicities while enhancing the therapeutic efficacy of PTX [7]. Although studies have demonstrated that PTX encapsulation in drug delivery systems based on liposomes enhanced the accumulation in the cancerous tissue while minimizing systemic toxicity, the only liposomal preparation approved for marketing is Lipusu®. This conventional liposome preparation made up of phospatidylcholine and phosphatidylglycerol (in a 9:1 molar ratio, respectively) was approved by the State Food and Drug Administration of China in 2006 for treatment of ovarian, breast, head and neck cancer, gastric and non-small cell lung carcinoma [4,8]. Conventional and long-circulating liposomes are important strategies used for PTX encapsulation; however, the major drawback of these systems is their limited long-term stability [9–14]. In addition, the therapeutic efficacy has shown to be similar to that of free drug. In this context, the development of an alternative formulation of PTX with good aqueous solubility, good stability, targeting delivery to tumor cells, and the ability to reduce side effects is still needed.

PTX, commonly known as an antimicrotubule drug, may promote cell death by different mechanisms. Evidence indicates that PTX alters specific intracellular signal transduction events, such as the activation of signaling proteins (Raf-1 and bcl-2), induction of the p-53 protein, expression of tumor necrosis factor, and activation of EGFR-tyrosine kinase complex [15]. Therefore, improving PTX intracytoplasmic delivery through its encapsulation in pH-sensitive liposomes can be an interesting strategy to enhance its therapeutic efficacy. pH-sensitive liposomes are designed to release encapsulated drug when a region of lower pH is attained. It is well known that the interstitial fluids of a number of tumors in humans and animals have an ambient pH varying between 6.0 and 7.0, whereas in normal tissue the extracellular pH is approximately 7.4. These liposomes also have the potential to undergo destabilization at the endosomal vesicles (pH values below 5.0), thereby preventing their degradation at the lysosomal level and promoting the release of the drug into the cytoplasm, in turn inducing cellular death [2,16]. The initial accumulation of pH-sensitive liposomes is achieved by passive targeting by means of the enhanced permeability and retention (EPR) effect. In order to further improve the binding and internalization of the liposomes, studies have reported the immobilization of molecules of folic acid on the surface of liposomes for the targeted delivery of anticancer agents [17,18]. It is well known that receptors for folic acid are frequently overexpressed in a wide variety of human tumors, especially breast cancer [19]. Therefore, in the present study, long-circulating and pH-sensitive PEG-coated (SpHL-PTX) and PEG-folate-coated liposomes containing PTX (SpHL-FT-PTX) were developed, and the physicochemical and morphological properties as well as the *in vitro* cytotoxic activity were evaluated. The cytotoxicity of these formulations was evaluated in folate receptor-positive human breast cancer cell lines, MCF-7 and MDA-MB-231, which express a low and high level of folate receptor, respectively [20]. Furthermore, in order to promote an increase in PTX encapsulated in the liposomes, formulations containing soy phosphatidylcholine (SPC) were also prepared.

2. Materials and methods

2.1. Materials

Paclitaxel was purchased from Quiral Quimica do Brasil S.A (Juiz de Fora, Brazil). Dioleoylphosphatidylethanolamine (DOPE), soy phosphatidylcholine (SPC), and distearoylphosphatidylethanolaminepolyethyleneglycol$_{2000}$ (DSPE-PEG$_{2000}$) were acquired from Lipoid GmbH (Ludwigshafen, Germany). Distearoylphosphatidylethanolaminepolyethyleneglycol$_{2000}$-folate (DSPE-PEG$_{2000}$-folate) was supplied by Avanti Polar (Alabaster, USA). Cholesteryl hemisuccinate (CHEMS) was supplied by Sigma Chemical Company (St. Louis, USA). Sodium chloride was obtained from Merck (Rio de Janeiro, Brazil).

For in vitro studies, the Roswell Park Memorial Institute medium (RPMI) 1620 was obtained from American Type Culture Collection (ATCC) (Manassas, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoluim bromide (MTT), and staurosporine were supplied from Gibco Life Technologies (Carlsbad, USA).

The cancer cell lines MCF-7 and MDA-MB-231 (human breast adenocarcinoma) cells were purchased from American Type Culture Collection (ATCC) (Manassas, USA). Cell line L929 (fibroblast from mouse) was kindly supplied by Professor Dr. Helen Rodrigues Martins (Laboratory of Parasitic Diseases, Universidade Federal dos Vales do Jequitinhonha e Mucuri, Diamantina, Brazil). All other chemicals and reagents used were of analytical grade.

2.2. Experimental design

The main optimization goal was to obtain liposomes with a diameter as small as possible, presenting simultaneously a homogeneous diameter distribution, maximal PTX encapsulation, and appropriate morphological features. In addition, liposomes containing PTX must be able to entrap a suitable amount of drug for a long time.

It is known that the lipide:drug ratio is an important parameter in the design of promising liposomal formulations for clinical application [21]. In order to study the influence of different PTX concentrations and lipid composition and concentration on the physicochemical properties of the liposomes, drug-to-lipid molar ratios equal to 0.06, 0.12, and 0.24 were evaluated. The liposomal formulation with suitable characteristics such as diameter, polydispersity index, zeta potential, encapsulation percentage, and morphology, as well as adequate pH-sensitivity was selected for subsequent studies. First, multifunctionalized liposomes were obtained by immobilization of folate on the lipid vesicle surface; then, the stability and cytotoxicity of this system were evaluated.

2.3. Liposome preparation

SpHL-PTX liposomes were made up of DOPE, CHEMS, and DSPE-PEG$_{2000}$ in a 5.7:3.8:0.5 molar ratio, respectively. Similarly, SpHL-PC-PTX liposomes were made up of SPC, DOPE, CHEMS, and DSPE-PEG$_{2000}$ in a 2.0:4.5:3.0:0.5 molar ratio, respectively. Both formulations were prepared by the lipidic hydration method described by Bangham et al. [22]. Briefly, chloroform aliquots of the lipids were transferred to round bottom flask, and a lipid film was obtained by evaporating the chloroform under reduced pressure. PTX was added to the lipid solution. Next, the lipid film was hydrated with a solution of NaOH at a CHEMS/NaOH (mol/mol) ratio of 1:1 to promote the complete ionization of the CHEMS molecules. Finally, 0.9% w/v NaCl solution was added, followed by vigorous shaking in a vortex, producing multilamellar liposomes. This preparation was immediately submitted to the high-intensity probe sonication (20% amplitude) for 5 min, in ice bath, using a high-intensity ultrasonic processor (R2D091109 model; Unique® Instruments, Indaiatuba, Brazil). Non-entrapped PTX was eliminated by centrifugation (Sigma 4k-15 centrifuge, Sigma Laborzentrifugen GmbH, Osterode, Germany) at 3000 rpm at 4 °C for 10 min. The empty liposomes (without drug) were also prepared as described above. For SpHL-folate-PTX (SpHL-FT-PTX), 0.05% of DSPE-PEG$_{2000}$-folate was added in the first step of the preparation of lipid film. Subsequent steps were conducted similarly as described above.