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Liquid formulation containing doxorubicin-loaded lipid-core nanocapsules: Cytotoxicity in human breast cancer cell line and *in vitro* uptake mechanism

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ABSTRACT

Cancer is a major public health problem in the world, being breast cancer the most frequent cancer affecting women. Despite advances in detection and treatment, mortality rates remain high. Therefore, new approaches for breast cancer treatments are necessary. In this study, our objective was to develop a liquid formulation containing doxorubicin-loaded lipid-core nanocapsules (DOX-LNC), to evaluate the *in vitro* antiproliferative activity and to determine the nanocapsules uptake by MCF-7 cells. Lipid-core nanocapsules (LNC), blank formulation, and DOX-LNC, proposed treatment, were prepared by self-assembling using the solvent displacement method. Hydrodynamic mean diameters (z-average) were respectively 191 ± 31 nm and 230 ± 23 nm presenting narrow size distributions. Drug content was 0.102 ± 0.029 mg mL^{-1} with an encapsulation efficiency higher than 90%. Formulations were applied to semiconfluent MCF-7 cells. After 24 h, LNC showed no cytotoxicity, while DOX-LNC showed an IC_{50} of 4.49 micromolar. After 72 h of incubation, DOX-LNC showed an IC_{50} of 1.60 micromolar demonstrating a sustained effect. The nanocapsules were internalized by endocytosis mediated by caveolin and by fluid phase endocytosis, which are active transport mechanisms. In conclusion, the liquid formulation containing DOX-LNC showed to be a promising product for the breast cancer treatment opening new avenues for further *in vivo* studies.

1. Introduction

Cancer is a major public health problem in the world despite the advances in either diagnosis or treatment in the past 40 years. Therefore, cancer mortality remains high [1]. To circumvent this issue, the development of treatments using nanotechnology, especially the development of novel carriers for cancer chemotherapy, has generated widespread interest [2]. In the last decades, the development of nanoparticle formulations have become a popular strategy for treating cancerous tumors [3]. Example of currently commercially available products based on nanotechnology are albumin-bound paclitaxel nanoparticles (Abraxane®), doxorubicin hydrochloride encapsulated in Stealth® liposomes (Doxil®) and amphotericin B encapsulated in liposomes (AmBisome®) [4], as well as irinotecan-loaded liposomes (Onivyde®), nanocrystalline suspension of dantrolene sodium (Ryanodex®), and paliperidone palmitate nanocrystals (Invega® Sustenna®) [5]. Other formulations containing doxorubicin, based on pegylated-liposomes and on poly(iso-hexyl cyanoacrylate)-nanoparticles, have been developed and are under clinical trials, phases I/II and III [6].

Liposomes, submicronic lipid vesicles, and synthetic polymer nanoparticles are able to carry small molecules and macromolecules into tissues and cells [7]. The supramolecular structure of those soft-nanocarriers are constituted of molecules and macromolecules interacting by weak bonds, such as hydrogen bonds, dipole-dipole interactions and London dispersion force, and, in some cases, by ionic chemical bonds. In particular, the interest of polymeric nanocapsules, a type of synthetic polymer nanoparticle containing an oily cavity surrounded by a polymer-wall, became exceptionally promising due to their high drug loading capacity and effective drug targeting [7–9]. Among the different polymeric nanocapsules, the lipid-core nanocapsules (LNC) are excellent nanocarriers to deliver antitumor drugs to their site of action [10]. Recently, additional studies demonstrated the *in vivo* effectiveness of methotrexate-loaded LNC [11] and indomethacin (drug and pro-drug)-loaded LNC [12] in treating

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glioblastoma, since LNC can act as a drug shuttle crossing the blood brain barrier to deliver the drug in brain tissue. The high efficiency in reducing the tumor has been observed after either intravenous or oral administration [12]. Furthermore, methotrexate diethyl ester-loaded LNC improved apoptosis induction, reverting resistance, in metastatic breast cancer MDA-MB-231 cells [13]. More recently, we demonstrated that LNC, prepared without any drug, presents a novel therapeutic mechanism further than a drug delivery system using SK-Mel-28 cells, as melanoma model [14]. Moreover, we also observed that the supramolecular structure of the nanocapsules, defined by the molecular and macromolecular materials interacting one to another, is relevant to the pharmacological effect of the formulation. Acetyl eugenol-loaded LNC, the drug-loaded formulation, has lower therapeutic effectiveness than LNC, the drug-unloaded one, likely because of the high interaction of acetyl eugenol with the nanocapsule polymer wall decreasing the crystallinity of the polymer [14].

Doxorubicin (DOX), an anthracycline antibiotic, is a well-known chemotherapy drug used in the treatment of a wide variety of cancers. Furthermore, the clinical application of DOX is limited by its adverse effects, which cumulative dose-dependent cardiotoxicity is the most serious among them. The nanoencapsulation of DOX leads to its accumulation at the site of action by an enhanced permeability and retention (EPR) effect limiting its biodistribution to healthy tissues [15–17]. Tumor treatments are conducted with DOX hydrochloride salt (water-soluble form of the drug), which is intravenously administered independent of the pharmaceutical composition, liposomal or solution [18].

Breast cancer is the most frequent cancer affecting women [1], and as a consequence MCF-7 cell line is the most common *in vitro* model in cancer studies [19]. Taking into account the ability of LNC to cross biological barriers including the oral absorption, we aimed to develop a liquid formulation containing doxorubicin-loaded LNC to determine its efficacy as antitumor agent using human breast cancer cell line (MCF-7) and the *in vitro* cellular uptake mechanism. Previous to the biological evaluations, the formulation was prepared by a self-assembling method and characterized in terms of particle sizing, drug content and encapsulation efficiency.

2. Material and methods

2.1. Materials

Doxorubicin hydrochloride, Span® 60 (sorbitan monostearate), poly(ε-caprolactone) (α,ω-dihydroxyl, Mn 10 kg mol^{-1}, Mw 14 kg mol^{-1}), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI·HCl), 4-(*N*,*N*-dimethyl)aminopyridine (DMAP) were obtained from Sigma-Aldrich (St Louis, MO, USA). Caprylic/capric triglyceride and polysorbate 80 were delivered by Delaware (Porto Alegre, Brazil). Solvents used were of analytical or pharmaceutical grades.

For cell culture studies, Dulbecco's modified Eagle's medium (DMEM low) was obtained from Sigma-Aldrich (St Louis, MO, USA) and fetal bovine serum (FBS) from Gibco (Grand Island, NY, USA). HEPES was purchased from Fluka and sodium bicarbonate, Fungisone® and Streptomycine from Sigma-Aldrich (St Louis, MO, USA), as well as 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA); azithromycin (AZT); methyl-β-cyclodextrin (CD); phenylarsine oxide (PheAso); sodium azide (AZD) and filipin III (FIII) used in determination of uptake mechanism. For confocal microscopy images, FluoroShield® from Sigma-Aldrich (St Louis, MO, USA) was used as a mounting medium. For cell viability study, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MMT) was obtained from Sigma-Aldrich (St Louis, MO, USA).

2.2. Methods

2.2.1. Neutralization of doxorubicin hydrochloride

Doxorubicin (freebase) was obtained as previously reported [20] by neutralization of doxorubicin hydrochloride following extraction. Doxorubicin hydrochloride was dissolved into ultrapure water at a concentration of approximately 1 mg mL^{-1}. The aqueous phase was poured into a separator funnel and chloroform (1 mL) was added. Trimethylamine (in molar excess) was added drop-by-drop using a Pasteur pipette. The separator funnel was capped for the liquid-liquid extraction. The chloroform phase turned to red indicating that doxorubicin (freebase) was extracted. The chloroform phase was collected and the extraction was repeated until the aqueous phase became colorless. The chloroform phases were combined and this organic phase was dried with anhydrous sodium sulfate. The organic phase was filtered and the solvent was removed using a rotary evaporator (Büchi, Switzerland) under reduced pressure. The residue was weight and dissolved in acetone (1 mL) to be used in the next step (preparation of doxorubicin-loaded LNC).

2.2.2. Preparation of doxorubicin-loaded LNC

Poly(ε-caprolactone) (0.100 g) and sorbitan monostearate (0.038 g) were dissolved in acetone (27 mL). Then, the solution was maintained at 40 °C to the addition of capric/caprylic triglyceride (0.160 mL) and DOX acetone solution (1 mL, obtained as described above). This organic solution was injected into an aqueous phase (53 mL) containing polysorbate 80 (0.077 g) at 40 °C. After 10 min, acetone was evaporated and the formulation concentrated under reduced pressure at 40 °C using a rotary evaporator (Büchi, Switzerland) until approximately 9 mL. The final volume was adjusted using ultrapure water to 10 mL in a volumetric flask. The formulation was named DOX-LNC. A formulation containing no drug, named LNC, was also prepared as described above omitting doxorubicin in the process.

2.2.3. Preparation of fluorescent-labeled LNC

In order obtain fluorescent LNC to be used in the uptake studies, a polymer-dye conjugate was obtained by esterification of PCL with rhodamine B as previously reported [21]. A solution of rhodamine B (0.154 g) in dry dichloromethane (10 mL) was added of EDCI·HCl (0.7344×10^{-5} g) under magnetic stirring at 0 °C. After 1 h, DMAP (0.4×10^{-4} g) was added. After 1 h under stirring, a solution of PCL (2.8 g) in dichloromethane (15 mL) was added into the medium. The reaction was maintained under inert atmosphere for 5 days at room temperature (18–22 °C). The polymer-dye conjugate (PCL-RhoB) was purified by liquid-liquid extraction and column chromatography. First, the reactional medium was extracted with 1.0 mol L^{-1} HCl (3 × 30 mL) in order to remove residues of EDC·HCl and DMAP, followed by the extraction with sodium bicarbonate saturated aqueous solution (2 × 30 mL). Finally, PCL-RhoB was purified by column chromatography using silica gel (70–200 mesh), as stationary phase, and a dichloromethane:methanol gradient (98:2 to 95:5, v/v), as eluent. The fluorescent-labeled nanoformulations were prepared as described above using 10% (w/w) of PCL-RhoB in a blend with PCL. The formulations were named LNC-RhoB and DOX-LNC-RhoB, respectively, omitting or not the drug in the process.

2.2.4. Laser diffraction analysis

The formulations were initially evaluated by laser diffraction using a Mastersizer® 2000 instrument (Malvern Instruments, Worcestershire, UK). Each sample (without any treatment) was directly inserted in the wet unit (Hydro 2000SM - AWM2002, Malvern) containing about 100 to 150 mL of distilled water, stirred at 1.500–2.500 rpm, at room temperature. Volume-weighted mean diameters ($d\,[4,3]_v$) and polydispersity (expressed by the measure of the distribution width, Span) were calculated by the Malvern software based on Eqs. (1) and (2), respectively.

$$d[4,3] = \frac{\sum_i^n d_{i\,v_i}^4}{\sum_i^n d_{i\,v_i}^3} \quad (1)$$