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Original article

Doxazosin nanoencapsulation improves its *in vitro* antiproliferative and anticlonogenic effects on breast cancer cells

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ABSTRACT

Doxazosin has been evaluated for the treatment of several types of cancer. Here, the antitumor effect of the nanoencapsulated form of doxazosin was evaluated in an *in vitro* model of breast cancer (MCF7 cell line). Doxazosin-loaded polymeric nanocapsules (DXZ-NC) were produced by interfacial deposition of preformed polymer with homogeneous aspect, spherical shape, mean diameter of about 130 nm, positive zeta potential (+5 mV), and encapsulation efficiency close to 35%. The Alamar Blue® assay and cell counting were carried out to assess cell viability and cell number, respectively. Mechanism of death was evaluated by Annexin/Propidium Iodide staining, while the long-term response was assessed using the clonogenic assay. Nuclear morphometric analysis was investigated using the NMA technique. A significant decrease in cell viability and clonogenicity was observed after the treatment with DXZ-NC when compared to the non-encapsulated drug. All treatments induced apoptosis as the main mechanism of toxicity. In conclusion, the nanoencapsulation of doxazosin improved its *in vitro* effects in MCF7 cells, without changing the mechanism of cell death underlying its toxicity. This approach was fundamental to reduce the long-term *in vitro* ability of the remaining tumor cells to form new colonies after the treatment, potentially reducing the risk of tumor recurrence.

1. Introduction

Cancer is still a significant worldwide health problem [1,2]. The urgency in the prospection of new anti-cancer agents, new formulations, and new drug delivery strategies is explained in view of the severe adverse effects of commonly used antitumor drugs, along with the increasing resistance of tumor cells to conventional chemotherapy [2]. Breast cancer is the second most frequent tumor type in the world after the lung cancer, and is the most common type of cancer in the female population [3]. Due to medical progress in the diagnosis and treatment, the survival rate for breast cancer has increased since 1990. However, it still is alarmingly high, since the disease is the most common cause of death among women in the world [3,4].

Doxazosin (DXZ) is a quinazolin compound classified as type 1 of alpha-adrenergic selective blockers. It has been used in the treatment of benign prostatic hyperplasia and as an anti-hypertensive [1,5,6]. This quinazolin is formed by benzene and pyrimidine, two fused aromatic rings that are well known for their biological activities due to their multiple pharmacophores [7,8]. Gefitinib and lapatinib are examples of drugs used to treat breast cancer, and both have this quinazolin in their structure [9].

DXZ has been also described as a potent inhibitor of cell growth and as an apoptosis inducer, with antiangiogenic effect on some cancer cell lines [1,10–13]. Different mechanisms of action underlying its cytotoxic effect has been described, in an adrenergic receptors-independent way. Hui et al. reported that DXZ induces apoptosis in breast cancer cells (MDA-MB-231 and MCF7) through the inhibition of both epidermal growth factor receptor (EGFR) and nuclear transcription factor (NF-kB), which is involved in the formation of new vessels [10]. Park et al. demonstrated an antiangiogenic effect for DXZ in human ovarian cancer. *In vitro* studies showed the inhibition of capillary induced by the vascular endothelial growth factor (VEGF). In addition, in an *in vivo* study

Abbreviations: CIS, cisplatin; DXZ, doxazosin; DXZ-NC, doxazosin-loaded nanocapsules; MCT, medium chain triglycerides; PE, plate efficiency; SF, survival factor; UNC, unloaded nanocapsules.

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using xenograft model, DXZ modulated angiogenesis through the inhibition of phosphorylation of protein kinase B (Akt) and mammalian target of rapamycin (mTOR), besides decreasing VEGF expression [11]. Staudacher et al. showed that DXZ promotes anti-proliferative effects in an *in vitro* study using glioblastoma cell lines (LNT-229 and U87MG) by blocking the cell cycle in G0/G1, having an apoptotic activity by suppressing hERG [12]. Gaelzer et al. also demonstrated that DXZ induces cell death and inhibits cell proliferation through caspase-3 activation and cell cycle arrest at the G0/G1 phase in glioblastoma cells (C6 and U138-MG). The mechanisms of action identified to explain this effects are the inhibition of the PI3 K/Akt pathway, with a reduction in Akt level, besides the upregulation of GSK-3β and p53 [13].

DXZ is currently marketed as its mesylate form. The main adverse events reported after its administration are dizziness, erectile dysfunction, dry mouth, prostate disorder, postural hypotension, cataracts, and abnormal renal function [14]. The administration of DXZ mesylate has been also associated with increased risk of heart disease, in ischemic patients [15]. Moreover, its cytotoxic effects on normal tissues such as digestive tract, heart and bone marrow are usually dose-limiting, thereby hampering its efficacy of chemotherapy [16]. Novel drug delivery systems as nanocarriers have been extensively reported to reduce adverse effect of different drugs [17].

Polymeric nanocapsules, as drug delivery systems, have attracted considerable attention in recent decades due to their ability to increase the therapeutic efficacy and also reduce adverse effects of several drugs [18]. These systems are designed to alter the pharmacokinetics and biodistribution of the encapsulated drug, acting as controlled drug release systems [19] and/or to overcome biological barriers [20]. Polymeric nanoparticles are drug carriers described as colloidal particles of mean diameter under 1 μm and a narrow particle size distribution [21]. This category of particles comprises nanocapsules and nanospheres, which differ in composition and structural organization. Nanocapsules are vesicular particles, whereas nanospheres consist of a matrix structure [21,22].

Regarding the controlled drug release approaches to improve therapeutic efficacy, studies have reported the development of tablets [23] and transdermal systems [24] to control DXZ release rate, mainly for the therapy of benign prostatic hyperplasia. However, the influence of its encapsulation in nanocarriers on cancer cells has not been studied yet. In this scenario, this study aimed to encapsulate DXZ in polymeric nanocapsules as a strategy to improve its antitumor effects on breast cancer cell line (MCF7). In order to evaluate the effects of the nanoencapsulation on *in vitro* drug efficacy, cell viability, mechanism of death induced, nuclear morphology and, as a long term assay, the ability of cells to form colonies were assessed.

2. Material and methods

2.1. Materials

DXZ mesylate was supplied by Delaware (purity grade 100%) (Porto Alegre, Brazil). Eudragit® RS100 polymer was obtained from Evonik Industries Corp. (Essen, Germany). Medium chain triglyceride (MCT) was purchased from Delaware (Porto Alegre, Brazil). Polysorbate 80 and acetone were acquired from Vetec (Rio de Janeiro, Brazil). HPLC-grade acetonitrile, acid acetic, and methanol were obtained from Tedia (São Paulo, Brazil). All reagents used were of pharmaceutical or HPLC grade and used as received.

Dulbecco's Modified Eagle's Medium (DMEM), penicillin/streptomycin, fungizone, trypsin/EDTA solution and DAPI were supplied by Sigma-Aldrich Co. Alamar Blue® was obtained from Invitrogen (Paisley, UK). Fetal bovine serum (FBS) was purchased from Gibco. Annexin V/Propidium Iodide apoptosis detection kit (sc-4252 AK) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Preparation of nanocapsule suspension

DXZ-loaded nanocapsules (DXZ-NC) [25] were produced (n = 3) by interfacial deposition of preformed polymer [26]. The polymer (Eudragit® RS100) (0.1 g) was dissolved in acetone (24.2 mL), under magnetic stirring for 30 min (at 37 °C), then the oil (MCT) (165 μL) and a DXZ methanolic solution (1.33 mg mL^{-1}, 3 mL) were added, composing the organic phase of the formulation. This organic phase was stirred for a further 10 min. After, the organic phase was injected in an aqueous phase composed of polysorbate 80 (0.077 g) and ultrapure water (54 mL), while stirring. After 10 min of stirring, methanol and acetone were removed under reduced pressure (at 40 °C) and the suspension was concentrated reaching a final volume of 10 mL (drug concentration of 0.4 mg/mL). Nanocapsules without drug (unloaded nanocapsules, UNC) were also prepared (n = 3) using the same method. Nanocapsule suspensions were kept at room temperature and protected from light until analysis.

2.3. Physicochemical characterization of formulations

2.3.1. Particle size, polydispersity index and zeta potential

Particle size was analyzed by laser diffraction. The formulation was added directly in the aqueous disperser compartment of the equipment (Mastersizer 2000®, Malvern, Worcestershire, UK). Volume-weighted mean diameter (*D*4,3) and polydispersity (Span) were also recorded. Mean diameter (Z-average) and polydispersity index (PDI) were analyzed by dynamic light scattering (ZetaSizer®, Nano-ZS Model ZEN 3600, Malvern, England) diluting the suspensions (500 times, v/v) in ultra pure water. In the same equipment, the zeta potential of the formulations was determined by electrophoretic mobility after dilution of the formulations (500 times, v/v) in 10 mM NaCl solution, previously filtered through a membrane filter (Millex HV PVDF 0.45 μm). For all techniques, the samples were analyzed in triplicate batches (n = 3).

2.3.2. Morphological analysis

The morphology of the nanocapsules was evaluated by transmission electron microscopy (TEM; JEM 1200 Exll operating at 80 K, Japan). The analyses were carried out at the Center of Electron Microscopy and Microanalysis (UFRGS, Brazil). A Formvar/Carbon (Electron Microscopy Sciences, USA) grid was used to deposit the aqueous suspension sample (1 μL) and negatively stained with uranyl acetate solution (2% w/v).

2.3.3. pH measurements

The pH of nanocapsule suspensions was determined at room temperature (25 ± 2 °C), immersing a calibrated potentiometer directly in the formulations (Digimed, DM-22, São Paulo, Brazil).

2.3.4. DXZ content and encapsulation efficiency

The amount of DXZ and its encapsulation efficiency in nanocapsules was determined by liquid chromatography (LC) according to a previously validated method [25]. The system consisted of a LC device (Shimadzu, Kyoto, Japan) equipped with a SIL-20A auto-injector, a DGU-20A5 degasser, a SPD-20AV UV detector, a LC-20AT pump, a CBM-20A5 system controller. A Phenomenex Gemini C18 column (250 × 4.6 mm; 5 μm, 110° A) was used as the stationary phase. The mobile phase run at isocratic flow rate (0.8 mL $minute^{-1}$) was composed of acetonitrile and water (80:20 v/v), acidified with acetic acid to pH 3.5. The mobile phase was filtered through a 0.45-μm cellulose acetate membrane