



Superiority of aromatase inhibitor and cyclooxygenase-2 inhibitor combined delivery: Hyaluronate-targeted versus PEGylated protamine nanocapsules for breast cancer therapy



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ABSTRACT

Despite several reports have revealed the beneficial effect of co-administration of COX-2 inhibitors with aromatase inhibitors in managing postmenopausal breast cancer; no nanocarriers for such combined delivery have been developed till now. Therefore, protamine nanocapsules (PMN-NCs) have been developed to co-deliver letrozole (LTZ) that inhibits aromatase-mediated estrogen biosynthesis and celecoxib (CXB) that synergistically inhibits aromatase expression. Inspired by the CD44-mediated tumor targeting ability of hyaluronate (HA), we developed HA-coated PMN-NCs (HA-NCs) via electrostatic layer-by-layer assembly. Moreover, multi-compartmental PEGylated phospholipid-CXB complex bilayer enveloping PMN-NCs (PEG-NCs) were designed for conferring biphasic CXB release from the phospholipid corona and oily core as well as enabling passive-targeting. The NCs demonstrated excellent stability, prolonged circulation and could be scaled up with the aid of spray-drying technology. Hemolysis, serum stability and cytotoxicity studies confirmed the superiority of combined LTZ-CXB nano-delivery. Mechanistically, the NCs especially HA-NCs and PEG-NCs demonstrated precious anti-tumor effects in vivo revealed as reduction in the tumor volume and aromatase level, increased apoptosis, as well as inhibition of VEGF, NF- κ B and TNF- α augmented by histopathological and immunohistochemical studies. Overall, our approach provided for the first time a potential strategy for targeted LTZ-CXB combined therapy of hormone-dependent breast cancer via singular nanocapsule delivery system.

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1. Introduction

Aromatase inhibitors (AIs) are successfully used in treatment of ER α -positive breast cancer by inhibiting the conversion of androgens to estrogens (Fabian, 2007). Unfortunately, resistance commonly occurs to AIs in patients with metastatic tumor. Therefore, combined anti-cancer therapy may be useful in preventing or delaying emergence of resistance (Leary and Dowsett, 2006). On another avenue, an important contribution

for COX-2 has been documented in the etiology of breast cancer (Arun and Goss, 2004). Prostaglandin E₂, whose synthesis is triggered by COX, increases aromatase gene expression and hence COX-2 inhibitors e.g. celecoxib (CXB) could inhibit aromatase expression and consequently reduce estrogen production in breast cancer cells (Díaz-Cruz et al., 2005; Rudner et al., 2010; Wong et al., 2014). Based on these data, the synergistic combination of CXB with AIs e.g. exemestane or letrozole (LTZ) in treatment of breast cancer was proved by several preclinical and clinical studies (Falandry et al., 2009).

Both LTZ and CXB have poor aqueous solubility (2.9 μ g/ml and 3–7 μ g/ml at 37 °C, respectively) hindering the intravenous (i.v.) administration of both drugs (Seedher and Bhatia, 2003; Siddiqua et al., 2014). Moreover, the oral formulations of LTZ (Femara[®]) and

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CXB (Celebrex[®]) offer uncontrolled delivery, lack specificity, often lead to poor patient compliance and result in systemic side effects following long treatment. These adverse effects include bone loss, deep vein thrombosis and hyper-cholesterolemia for LTZ whereas CXB may cause thromboembolism and cardiovascular risk. In addition, these drugs are also rapidly cleared from circulation, which can reduce their therapeutic concentration at tumor site (Davies et al., 2000; Haynes et al., 2003). Therefore, novel tumor-targeted delivery systems are needed to enable i.v. administration and enhance tumor targeting resulting in reduced toxicity and improved efficacy of both drugs.

HA-targeted LTZ-loaded PLGA-PEG nanoparticles have demonstrated more powerful tumor growth inhibition in LTZ-resistant breast tumor animal model compared to the non-targeted nanoparticles (Nair et al., 2011). Similarly, modifying liposomes co-encapsulating epirubicin and CXB by a human immunodeficiency virus peptide (PTD_{HIV-1}) resulted in enhanced penetration and improved anticancer activity in invasive breast cancer bearing mice (Ju et al., 2014). However, no combined delivery of AI and COX-2 inhibitor nano-sized formulation was reported till now. Therefore, a combined nanocarrier for delivery of both LTZ and CXB should be developed to benefit from: (a) The synergistic aromatase inhibiting activity of CXB, (b) The broad anti-cancer activity of CXB mediated via multiple mechanisms other than aromatase blocking, (c) Finally, CXB can reduce the emergence of LTZ resistance (Leary and Dowsett, 2006). This combined delivery would help reduce the dose, decrease side effects and enhance therapeutic activity of LTZ in breast cancer therapy.

Recently, natural polymeric nanocarriers particularly those based on proteins and peptides have attracted great attention as colloidal vehicles for tumor-targeted delivery of anticancer drugs (Elgindy et al., 2012). Protamines (PRM) are biodegradable arginine-rich peptides of approximately 4 kDa, which occur naturally in sperm and can be extracted from fish testicles (Elzoghby et al., 2015a; Elzoghby et al., 2016). PRM has been long used for reversal of heparin toxicity or as excipient in insulin preparations. Furthermore, PRM has been exploited for gene delivery applications (Pali-Schöll et al., 2013) in addition to its potential use in brain and skin drug delivery (Choi et al., 2012a; Dhami et al., 2014).

Therefore, in this study, we propose for the first time up to our knowledge, the co-delivery of LTZ and CXB via protamine-based nanocapsules (PRM-NCs). This formula is intended to overcome the high lipophilicity of both drugs thus enables their i.v. administration. Moreover, for active targeting, PRM-NCs were electrostatically coated with HA. HA binds specifically to CD44 (cluster of differentiation 44) and RHAMM (receptor for hyaluronan-mediated motility) over-expressed in many types of cancer cells, demonstrating enhanced internalization via receptor-mediated endocytosis (Choi et al., 2012b). On the other hand, for passive targeting, PRM-NCs were enveloped by PEGylated phospholipid-CXB complex bilayer. The hydrophilic PEG surrounding PRM-NCs can protect them from reticuloendothelial system (RES) uptake and therefore increases their circulation time and enables selective extravasation at the leaky tumor vasculature (Suk et al., 2016). More importantly, the presence of long hydrophobic acyl chains of the phospholipid bilayer can solubilize CXB via complex formation. This multi-compartmental nanocarrier is intended to confer biphasic CXB delivery where the phospholipid complex bilayer will provide enhanced CXB release followed by slow release from the oily core. Thus, the early released CXB can begin inhibiting aromatase expression to reduce the enzyme amount that LTZ directly inhibits its activity and thus reducing LTZ dose whereas the slowly released CXB can offer long-term enzyme inhibition. The three developed NCs were thoroughly investigated for in vitro and in vivo parameters to explore the influence of different surface

characteristics on their efficacy. Meanwhile, the anti-tumor efficacy of the combined drug nano-delivery was compared with the free drug combination to prove the superiority of nano-encapsulation. Finally, a scale-up approach was applied as a step towards industrial development of dual drug-loaded PRM-NCs.

2. Experimental section

2.1. Materials

Letrozole (LTZ) was purchased from Xi'an Natural Field Bio-Technique CO., LTD. (China) while Celecoxib (CXB) was obtained as a gift sample from Amriya Pharmaceutical Industries, PHARCO Corporation (Alexandria, Egypt). Protamine sulfate (PRM), fetal bovine serum (FBS), 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), D-mannitol, lactose monohydrate and maltodextrin, Fluorescein isothiocyanate (FITC), Dimethyl sulfoxide (DMSO), Ethylene diaminetetraacetic acid (EDTA), Triton X100, Haematoxylin solution, Eosin solution and Canada balsam were purchased from Sigma-Aldrich (St. Louis, USA). Oils (Capryol 90, Capryol PGM C and Lauroglycol 90) were kindly supplied by Gattefosse (Lyon, France). Fat-free soybean phospholipids with 75% phosphatidylcholine (Lipoid S75) and N-(Carbonyl-methoxy-polyethyleneglycol-2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine, PEG-2000-DSPE were kindly provided by Lipoid (Ludwigshafen, Germany). Hyaluronic acid (Mwt 50,000–1,000,000 Da) was purchased from Baoding Faithful Industry Co., Ltd. (China). Polyoxyethylenesorbitanmonooleate (Tween 80) was purchased from (Riedel-de Haën, Germany). Poly(ethylene glycol) 400 (PEG-400) was supplied by El-Pharonia Pharmaceuticals Alexandria, Egypt. Sodium lauryl sulphate (SLS) and absolute ethanol were purchased from ADWIC, El-Nasr Pharmaceutical Chemicals Co. (Cairo, Egypt). Methanol HPLC grade was purchased from JT Baker (Phillipsburg, NJ, USA).

2.2. Preparation of dual drug-loaded PRM-NCs

PRM-NCs were prepared by a two-stage polymer-coating technique (Mora-Huertas et al., 2010; Oyarzun-Ampuero et al., 2013). The method involves deposition of the cationic PRM corona onto the anionic nanoemulsion core mainly by electrostatic interaction. First, 10 mg of LTZ and 20 mg of CXB were dissolved in 0.75 ml of Lauroglycol[®] 90 at 50 °C then mixed with 10 ml ethanolic solution containing 50 mg Lipoid[®] S75. This organic solution was poured, under moderate magnetic stirring, into 50 ml of aqueous solution containing Tween[®] 80 (0.2% w/v). The resulting mixed phase immediately turned milky as a result of the formation of nanoemulsion due to the diffusion of ethanol towards the aqueous phase. The solvents were then evaporated from NC suspension under reduced pressure in a rotary evaporator (Rotavapor[®] R-300, Büchi, Switzerland) at 45 °C and 50 rpm to a final volume of 10 ml. Finally, 2.5 ml of the nanoemulsion was incubated with 1 ml of 1% w/v PRM aqueous solution under gentle magnetic stirring for 30 min leading to the formation of PRM-NCs.

For preparation of HA-coated dual drug-loaded PRM-NCs (HA-NCs), different volumes of 0.3% w/v HA aqueous solution was gradually added to 2.5 ml of the PRM-NCs solution under mild magnetic stirring for 30 min. HA coating was achieved by the electrostatic interaction between the cationic PRM and the anionic HA (Mora-Huertas et al., 2010).

For preparation of PEGylated phospholipid-CXB complex bilayer enveloping dual drug-loaded PRM-NCs (PEG-NCs), film-hydration method was first used for preparation of PEGylated phospholipid-CXB complex (Khan et al., 2013). Briefly, 1:2 molar ratio of CXB (10 mg) and phospholipids (Lipoid[®] S75 and DSPE-PEG 2000 in 2:1 w/w) were dissolved in 10 ml mixture of

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