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Original article

Tretinoin-loaded lipid-core nanocapsules overcome the triple-negative breast cancer cell resistance to tretinoin and show synergistic effect on cytotoxicity induced by doxorubicin and 5-fluororacil

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ABSTRACT

Nanostructured drug delivery systems have been extensively studied, mainly for applications in cancer therapy. The advantages of these materials include protection against drug degradation and improvement in both the relative solubility of poorly water soluble drugs as in targeting of therapy, due to the enhanced permeability and retention effect on tumor sites. In this work, we evaluate the antitumor activity of tretinoin-loaded lipid core nanocapsules (TT-LNC) in a tretinoin-resistant breast cancer cell-line, MDA-MB- 231, as well as the synergistic effect of combination of this treatment with 5-FU or DOXO. The inhibition of cell growth was assayed by MTT reduction. Live/Dead assay and DAPI staining evaluated cytotoxicity. Apoptosis was evaluated by Annexin V-PE/7AAD and the effect of chronic exposure was evaluated by colony formation assay. TT-LNC reduced the cell viability even at lower concentrations (1 μM) and displayed synergistic effect with 5-FU or DOXO on cytotoxicity and colony formation inhibition. Our work shows a possibility of using nanocapsules to improve the antitumoral activity of TT for its use either alone or in combination with other chemotherapeutic drugs, especially considering the chronic effect.

1. Introduction

Breast cancer is the second most common type of cancer in the world and the most common among women [1]. Breast cancer can be classified in different subtypes, according to the molecular and clinical characteristics of cancer cells [2]. At least five distinct molecular breast cancer intrinsic subtypes have been identified on the basis of gene expression profiling, which include luminal A, luminal B, HER2-enriched, basal-like and claudin-low tumors, beyond the normal breast-like group [3]. Claudin-low tumors seems to originate in long-lived mammary tissue stem cells and have poor prognostics features. These type of tumors presents low expression of luminal differentiation markers, high enrichment for epithelial-to-mesenchymal transition markers, immune response genes and cancer stem cell-like features [2,4]. The majority of claudin-low tumors are estrogen receptor (ER)-negative, progesterone receptor (PR)-negative and epidermal factor growth 2 (HER-2)-negative and are part of group known as triple-negative breast cancer (TNBC), which predominantly include some types of basal-like and, more rarely, other subtypes of tumors [4,5]. TNBC are invasive breast cancers, generally of a higher grade, highly resistant to chemotherapy, very proliferative and have the worst survival rates [6,7]. Patients with TNBC are more likely to develop visceral metastases and presents higher recurrence rates of disease and death compared to patients with another subtypes of breast cancer [8,9].

TNBC and claudin low-tumor subtype are less differentiated and difficult to treat. Evaluations of therapeutic effects of novel anti-tumoral drugs or innovative formulations of consolidate drugs are carried out by *in vitro* screening using different cell lines, as triple-negative and

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claudin-low subtype MDA-MB-231. [9–11]. These cells are poorly differentiated, estrogen receptor negative, highly metastatic, invasive, have mutant p53 and was described as been resistant or poorly responsive to retinoic acid therapy [12,13].

Retinoic acid, as well known as tretinoin (TT) is a vitamin A derivative which has been extensively studied as an anticancer agent because of its effect on cell differentiation and growth [14,15]. Its action takes place through interaction with nuclear receptors, called retinoic acid receptor (RAR) [16]. These receptors have different isoforms, such as RARα, β and γ. By binding to retinoid receptors, these compounds regulate expression of several genes involved in many cellular processes, especially those involved with cell cycle control [17]. Considering that the cytotoxic effect of TT is not expressive, its clinical use often occurs associated with the use of other therapeutic agents with additive or synergistic action. In vitro evaluations of TT association with different chemotherapeutic agents has shown interesting effects on cell growth and clonogenic potential inhibition, as well as apoptosis induction in several types of cancer cells [11,18,19].

Nanoformulations, including nanocapsules and nanoemulsions, have been extensively studied to improve the delivery of poorly water soluble drugs, as TT, as well as, the protection of this drugs against degradation [19–21]. Tretinoin-loaded lipid-core nanocapsules (TT-LNC) were previously described as having anticancer activity against lung cancer and myeloid leukemia cells [22,23]. This formulation overcame the resistance of human lung cancer cells A549 to TT treatment, with apoptosis induction, cell growth inhibition, induction of cell cycle arrest and up-regulation of p21 expression [22]. This mechanism seems to be related to better intracellular delivery of the drug. Considering the low responsiveness of the triple-negative breast cancer cells to tretinoin treatment, our objective was to evaluate the effect of the acute and chronic exposure of triple-negative breast cancer cell lines, MDA-MB-231 to tretinoin-loaded lipid-core nanocapsules. Additionally, we evaluate the possible synergistic effect of this formulation with the effects of consolidate antineoplastic drugs, 5-fluororacil (5-FU) and doxorubicin (DOXO).

2. Material and methods

2.1. Nanoformulations procedures

TT-LNC were prepared by interfacial deposition of polymer, using poly(ε-caprolactone) (PCL) as a biodegradable polymer [22,23]. An acetone solution (67 mL), containing 1% (w/v) of polymer, 0.77% (w/v) of sorbitan monostearate, 3.3% (v/v) of caprylic/capric triglyceride mixture and 0.05% (w/v) of tretinoin was injected in an aqueous phase containing 0.77% (w/v) of polysorbate 80, under moderate magnetic stirring for 10 min. Evaporation under reduced pressure at 40 °C was carried out to remove acetone and concentrate the aqueous phase to achieve 25 mL of nanocapsules suspension. Blank lipid-core nanocapsules (LNC) were prepared without the addition of the drug into the acetone solution. All preparations were kept protected from the light during all the time. The nanocapsules were characterized as previously described [23]. In the present study, we confirm the following characteristics: particle size, polydispersity index, zeta potential and drug content for each batches.

2.2. Cell culture

Cells were obtained from the Rio de Janeiro Cell Bank (PABCAM, Federal University of Rio de Janeiro, RJ, Brazil). The invasive, triple-negative, claudin-low subtype breast cancer cell line, MDA-MB-231 was cultured in Leibovitz's L15 medium, supplemented with 0.2 g/L of sodium bicarbonate and 10% of fetal bovine serum at a controlled atmosphere of 37° without CO_2. The experiments were performed with cells in the logarithmic phase of growth.

2.3. Proliferation and viability assay

MDA-MB-231 cells were seeded at a density of 2×10^4 cells/well into a 96-well plate, and allowed to adhere for 24 h. Then, cells were treated with different concentrations (0.1; 1; 2; 4 or 6 μM) of tretinoin (TT) diluted at DMSO, tretinoin-loaded lipid-core nanocapsules (TT-LNC) and relative amounts (volume) of blank lipid-core nanocapsules, without TT (LNC) for 24, 48 and 72 h. After these periods, cells were washed twice with phosphate-buffered saline (PBS; Gibco®, Carlsbad, USA) and 5 mg/mL of MTT solution was added to each plate and remained in contact with cells for 3 h at 37 °C. The medium was removed and 200 μL of DMSO was added to each well, in order to dissolve formazan crystals using a shaker for 20 min at 150 rpm. The absorbance of each well was read on a microplate reader at a wavelength of 492 nm. The inhibition (%) of cell proliferation was determined as follows: growth inhibition rate (%) = [1-(Abs_{492} treated cells/Abs_{492} control cells)] x 100. The obtained results are a mean of three independent experiments in triplicate for each experiment. Control cells were considered the cells that were not submitted to any treatment.

To evaluate the synergistic effect between TT or TT-loaded nanoformulations, 5×10^3 cells/well into 96 well-plate were treated for 72 h with 1 μM of TT, TT-LNC or LNC and then the medium was replaced for a medium with 1 μM of doxorubicin (DOXO) or 40 μM of 5-fluororacil (5-FU) for additional 48 h [24]. After this period, the cell viability was evaluated as described by MTT reduction method. To determine if the antitumor effects obtained with TT-LNC and DOXO or 5-FU combinations were synergistic, we calculated the combination index (CI) according to the Chou-Talalay (CI > 1.1, antagonism; CI = 0.9–1.1, additive effect; CI = 0.2–0.9, synergism; CI < 0.2 strong synergism) [25].

2.4. Live and dead assay

2×10^4 cells were seeded in 96-well plate and allowed to adhere for 24 h. After, cells were treated with 1 or 6 μM of TT, TT-LNC, as well as the correspondent volume of LNC for 72 h. At the end of incubation period, cells were washed and stained using the LIVE/DEAD® Viability/Cytotoxicity Assay Kit (Invitrogen). Live cells were able to take up calcein and could be analyzed by green fluorescent light emission (488 nm). Ethidium bromide homodimer diffuses through the now permeable membrane of dead cells and binds to DNA, which was detected by the red fluorescent signal (546 nm). The LIVE/DEAD assay was analyzed with a fluorescence microscope Olympus IX71 (Olympus Optical Co., Tokyo, Japan) by multicolor imaging. The recorded images were analyzed using CellˆF software (Cell, Olympus, Tokyo, Japan). The cell death rate was determined as follows:

Cell death rate (%) = dead cells (red cells)/total cells (red and green cells)

2.5. Clonogenic assay

To evaluate the effect of the chronic exposure to formulations, a clonogenic assay was performed seeding 400 cells per well in a 6-well plate and allowed to adhere for 24 h in 2 mL of Leibovitz's L15. Then, 1 μM of TT, TT-LNC, as well as the correspondent volume of LNC were added to plates and cells were incubated at 37 °C without CO_2 for additional 10 days. The control cells were considered the cells that were incubated in medium without any treatment. After the period, cells were washed with PBS, fixed in methanol:acetone 1:1 for 5 min and stained with crystal violet for 5 min. The percentual of colony formation was calculated dividing the number of colonies formed in the treatments group by the number of colonies formed in the control group. An additional clonogenic assay was performed exposing the cells after attach to the plate to 1 μM of TT or TT-LNC, as well as the