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Research paper

Encapsulation in lipid-core nanocapsules overcomes lung cancer cell resistance to tretinoin

Eduarda Schultze [a], Aline Ourique [b], Virginia Campello Yurgel [a], Karine Rech Begnini [a], Helena Thurow [a], Priscila Marques Moura de Leon [a], Vinicius Farias Campos [a], Odir Antônio Dellagostin [a], Silvia R. Guterres [b], Adriana R. Pohlmann [b,c], Fabiana Kömmling Seixas [a], Ruy Carlos Ruver Beck [b,*], Tiago Collares [a,*]

[a] Programa de Pós-Graduação em Biotecnologia (PPGB), Grupo de Pesquisa em Oncologia Celular e Molecular, Biotecnologia/Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas, Pelotas, Brazil
[b] Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil
[c] Departamento de Química Orgânica, Instituto de Química, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

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ABSTRACT

Tretinoin is a retinoid derivative that has an antiproliferative effect on several kinds of tumours. Human lung adenocarcinoma epithelial cell lines (A549) exhibit a profound resistance to the effects of tretinoin. Nanocarriers seem to be a good alternative to overcome cellular resistance to drugs. The aim of this study was to test whether tretinoin-loaded lipid-core nanocapsules exert an antitumor effect on A549 cells. A549 cells were incubated with free tretinoin (TTN), blank nanocapsules (LNC) and tretinoin-loaded lipid-core nanocapsules (TTN–LNC). Data from evaluation of DNA content and Annexin V binding assay by flow cytometry showed that TTN–LNC induced apoptosis and cell cycle arrest at the G1-phase while TTN did not. TTN–LNC showed higher cytotoxic effects than TTN on A549 cells evaluated by MTT and LIVE/DEAD cell viability assay. Gene expression profiling identified up-regulated expression of gene p21 by TTN–LNC, supporting the cell cycle arrest effect. These results showed for the first time that TTN–LNC are able to overcome the resistance of adenocarcinoma cell line A549 to treatment with TTN by inducing apoptosis and cell cycle arrest, providing support for their use in applications in lung cancer therapy.

1. Introduction

Retinoids are metabolites of vitamin A that play many roles in the body. Retinoid analogues and derivatives have been extensively investigated as promising anticancer agents due to their antiproliferative and prodifferentiation effects [1–4]. Tretinoin (TTN), also known as all-*trans* retinoic acid, is a naturally occurring retinoid that plays an essential role in regulation of differentiation, growth, and development of normal and malignant epithelial cells in various tissues [5]. TTN and its derivatives have been recognised as a group of cancer chemopreventive and therapeutic agents [6]. It has been used as an effective agent to induce remission in patients with acute promyelocytic leukaemia [7]. This compound also has effect on other types of tumours such as solid tumour [8,9], brain tumour stem cells [10] and squamous cell carcinoma [11]. The mechanism of action is mainly through regulation of gene expression by nuclear receptors, known as retinoic acid receptors (RAR), which have three subunits called RARα, β and γ [4]. RARs form heterodimers with another type of nuclear receptor, retinoid X receptors (RXRs) and bind to retinoic acid response elements (RAREs) in the promoter region of target genes [6]. Tretinoin binds to the RAR subunit of heterodimer RAR–RXR and induces transcription. These receptors can also interact with other pathways independently of interaction with RAREs [12].

Lung cancer is a heterogeneous disease with two main subtypes: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC can be subdivided into adenocarcinoma, squamous-cell lung carcinoma, and large cell lung carcinoma. The treatment for this type of cancer depends on the stage of disease and includes surgery, radiotherapy and chemotherapy.

Abbreviations: LNC, lipid-core nanocapsules; TTN, tretinoin; TTN–LNC, tretinoin-loaded lipid-core nanocapsules.

* Corresponding authors. Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Av. Ipiranga, 2752, Porto Alegre, 90610-000, RS, Brazil. Tel.: +55 5133085411 (R.C.R. Beck). Universidade Federal de Pelotas, Campus Universitário s/n, Capão do Leão, CEP 96010-900 RS, Brazil. Tel.: +55 5332757350 (T. Collares).
E-mail addresses: ruy.beck@ufrgs.br (R.C.R. Beck), collares.t@gmail.com (T. Collares).

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TTN has aroused a deep interest as a potential therapeutic agent for lung cancer [13,14]. However, many NSCLC exhibited a profound resistance to the effects of tretinoin [15]. This resistance may be related to suppression of RARβ2 and RARβ1′ expression as well as to other mechanisms including the intracellular delivery of tretinoin [16–18]. One strategy to overcome tretinoin resistance could be to enhance cellular uptake of this compound by its nanoencapsulation [16,19].

TTN incorporated in DOTAP/cholesterol liposomes showed a potent cytotoxic effect on A549 cells, which are insensitive to the effects of free TTN [16]. This result was appointed to increased internalisation of TTN promoted by the cationic liposomes. Further studies have investigated the benefits provided by incorporation into liposomes or other drug delivery systems compared to the action of free TTN [20]. However, there are no reports on the use of tretinoin lipid-core nanocapsules as a strategy to improve the antitumor effect on lung cancer cells.

Tretinoin loaded nanocapsules were reported in 2008 as having a significant protection of the drug against UVC radiation. However, the formulation showed poor stability during storage, dropping the drug content below 90% after 1 month of storage [21]. To improve the physicochemical stability of tretinoin-loaded polymeric nanocarriers, our group changed the type of nanocapsules, preparing a lipid-core nanocapsule containing TTN. Lipid-core nanocapsules are polymeric nanocapsules presenting a core composed of a dispersion of a liquid lipid, capric/caprylic triglyceride, and a solid lipid, sorbitan monostearate. In addition, these formulations (nanocapsules and lipid-core nanocapsules) are aqueous systems, allowing their administration by different routes and had low viscosity, which is desirable for a formulation for parenteral use [22]. These properties are very important from the pharmaceutical development point of view, since in some formulations the use of organic solvents or toxic surfactants is necessary to formulate hydrophobic drugs, such as TTN, in aqueous systems.

Considering all our previous results regarding the feasibility of TTN nanoencapsulation in aqueous dispersions, the aim of this study was to test if TTN–LNC would be able to overcome the resistance of human lung adenocarcinoma epithelial cell line A549 to TTN treatment.

2. Materials and methods

2.1. Preparation of nanocapsules

TTN–LNC were prepared as described before [22] by interfacial deposition of polymer, using poly(ε-caprolactone) at 1% (w/v) as a biodegradable polymer. Twenty-five milligrams 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 were dissolved in 67 mL of acetone. This organic solution was added to 134 mL of an aqueous phase containing 0.77% (w/v) of polysorbate 80 under moderate magnetic stirring for 10 min. Acetone was removed and the aqueous phase concentrated by evaporation at 40 °C under reduced pressure to obtain 25 mL. The concentration of tretinoin was set at 0.5 mg/mL. At higher concentration, simultaneous microparticles are formed due to the overconcentration of the drug, according to our preliminary studies (data not shown). Blank lipid-core nanocapsules (LNC) were prepared in a similar way, but without the addition of the drug into the organic phase. All preparations were kept protected from light at all times.

2.2. Characterisation of nanocapsules

Characterisation of nanocapsules was previously described for three independent batches [22]. In the present study, we confirm the following characteristics: particle size, polydispersity index, zeta potential and drug content. Particle size and the polydispersity index (PDI) were analysed by photon correlation spectroscopy (PCS) (Zetasizer® Nanoseries ZEN3600, Malvern Instruments, Worcestershire, England) after adequate dilution (1:500, v/v) of an aliquot of the suspension in filtered water (0.45 μm, Millipore, Bedford, USA). The zeta potential (ζ-potential) (Zetasizer® Nanoseries ZEN3600, Malvern Instruments, Worcestershire, England) measurements were performed at 25 °C according to the electrophoretic mobility principle after diluting (1:500, v/v) the samples with a filtered (0.45 μm, Millipore, Bedford, USA) 10 mM NaCl aqueous solution. Drug content was evaluated by HPLC according to the method previously validated [22].

2.3. Cell culture

The human lung adenocarcinoma cells (A549) were obtained from the Rio de Janeiro Cell Bank (PABCAM, Federal University of Rio de Janeiro, RJ, Brazil). They were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% foetal bovine serum (FBS), purchased respectively from Vitrocell Embriolife (Campinas, Brazil) and Gibco (Grand Island, NY, USA). Cells were grown at 37 °C in an atmosphere of 95% humidified air and 5% CO_2. The experiments were performed with cells in the logarithmic phase of growth.

2.4. Determination of cytotoxicity by MTT assay

The viability of the A549 cell line was determined by measuring the reduction of soluble MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) to water insoluble formazan [23]. A549 cells were seeded on a 96-well cluster dish at a density of 1×10^4 cells per well and grown at 37 °C in a 5% CO_2 atmosphere. Twenty-four hours later, cells were incubated with a medium containing tretinoin (TTN), blank lipid-core nanocapsules (LNC) or tretinoin-loaded lipid-core nanocapsules (TTN–LNC) at various concentrations (1, 5, 10 and 20 μM) for 24, 48 and 72 h at 37 °C. After these periods, cells were washed twice with phosphate-buffered saline (PBS; Gibco®, Carlsbad, USA); 5 mg/mL of MTT solution was added to each well and cells were incubated for 3 h at 37 °C in 5% CO_2. The medium was removed and then 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)] × 100 [24]. The obtained results are a median of three independent experiments in triplicate for each experiment.

2.5. Viability assessment and LIVE/DEAD assay

The LIVE/DEAD cell viability assay (Invitrogen™, Carlsbad, USA) was conducted following the manufacturer's instructions. Live cells were able to take up calcein and could be analysed 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 analysed with a fluorescence microscope Olympus IX71 (Olympus Optical Co., Tokyo, Japan) by multicolour imaging. After excitation at 480 nm and emission at 510 nm, the fluorescent images were stored as TIFF files using a digital camera (Olympus, Tokyo, Japan) attached to a fluorescence microscope (DP 12; IX71; Olympus, Tokyo, Japan). The recorded images were analysed using Cell^F software (Cell^F, Olympus, Tokyo, Japan). The data were expressed as the mean ± SEM of