Colloids and Surfaces B: Biointerfaces xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Anticancer activity of all-*trans* retinoic acid-loaded liposomes on human thyroid carcinoma cells

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ARTICLE INFO

Article history:
Received 21 July 2016
Received in revised form 22 October 2016
Accepted 25 October 2016
Available online xxx

Keywords:
All-*trans* retinoic acid (ATRA)
Liposomes
Human thyroid carcinoma
Drug delivery systems

ABSTRACT

All-*trans* retinoic acid (ATRA) is an anti-tumor compound, exerting different anti-cancer effects on different types of cancer cells. Unfortunately, retinoids are also characterized by certain side effects following systemic administration, such as the burning of skin and general malaise. The highly variable degree of bioavailability of ATRA plus its tendency to induce its own destruction through metabolic degradation following oral treatment necessitate the development of alternative formulations. The aim of this work is to evaluate the physico-chemical properties of unilamellar, ATRA-containing liposomes and to investigate the cytotoxic activity of this potential nanomedicine on human thyroid carcinoma cells. Liposomes made up of DPPC/Chol/DSPE-mPEG2000 (6:3:1 molar ratio), characterized by a mean diameter of ~200 nm, a polydispersity index of 0.1 and a negative surface charge, were used as ATRA-carriers and their antiproliferative efficacy was investigated in comparison with the free drug on three different human thyroid carcinoma cell lines (PTC-1, B-CPAP, and FRO) through MTT-testing. The liposomes protected the ATRA against photodegradation and increased its antiproliferative properties due to the improvement of its cellular uptake. ATRA-loaded liposomes could be a novel formulation useful for the treatment of anaplastic thyroid carcinoma.

1. Introduction

Retinoids belong to the family of polyisoprenols, which are lipids that include vitamin A (retinol) and are structurally related to it [1]. They fulfill crucial functions in the human body, such as cell proliferation and differentiation, bone tissue growth, immune function regulation, and tumor-suppressor gene expression [2,3].

Retinoids are used in the treatment of many diseases and are efficacious in the treatment of different dermatological conditions (inflammatory skin disorders, skin cancers, disorders concerning increased cell turnover, photoaging, acne and psoriasis) [4–6]. They are important for embryonic development and maintenance of the differentiated tissues and hence their property of inducing cell differentiation makes them suitable compounds for use in anticancer therapy. For this reason, a number of studies have investigated the potential application of retinoids in cancer treatment [7–9]. The antitumor effects of retinoids are associated with a direct antiproliferative property, as demonstrated by *in vitro* testing on different cell lines [10,11]. All-*trans* retinoic acid (ATRA) brings about a significant remission of different types of tumors [1,12,13] thanks to the regulation of gene expression through the ATRA-dependent activation of retinoic acid receptors (RAR) and retinoid X receptors (RXR) on the nuclear membranes of cancer cells; this leads to growth inhibition, differentiation and apoptosis [14,15].

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http://dx.doi.org/10.1016/j.colsurfb.2016.10.052
0927-7765/

From an oncological point of view, differentiation therapy is an approach useful for inducing malignant reversion, which is based on the concept that cancer cells can be blocked at an immature or less differentiated state, causing them to lose the ability to control their own growth and multiply at an abnormally fast rate [16,17]. Although differentiation therapy does not induce the death of cancer cells, it does restrain their growth and allows the application of more conventional therapies (such as chemotherapy) to eradicate the malignant cells.

The difficulty of using retinol and its derivatives in cancer therapy is related to the extreme lability of these compounds, while retinol and its oxidation products, such as retinal and retinoic acid, are very unstable hydrophobic compounds in the presence of oxygen, acidic environments and exposure to light [1]. A useful strategy for increasing the stability of retinoids is their encapsulation in drug delivery systems, which should be able to protect them from external agents [18,19]. For example, liposomes (self-assembled vesicles which can entrap hydrophilic, lipophilic and amphiphilic substances) are able to modulate the biopharmaceutical properties of the encapsulated compounds [20–22] and to improve the stability of the entrapped drug [23], even against photodegradation phenomena [24].

Aronex Pharmaceuticals realized an ATRA-containing liposome formulation (Atragen®), made up of dimyristoyl phosphatidylcholine (DMPC) and soybean oil, for the treatment of acute promyelocytic leukemia. This formulation is able to avoid clearance by hepatic microsomes, which normally occurs in the case of free (non-encapsulated) ATRA. Atragen® also demonstrated a lower degree of *in vivo* systemic toxicity than the free form of ATRA [25,26]. Unfortunately, the FDA did not approve this formulation because "Aronex data did not establish an identifiable population of patients who need tretinoin and cannot use the oral formulation" (http://listofcompanies.co.in/it/aronex-pharmaceuticals-inc/). To the best of our knowledge a liposomal formulation containing ATRA has not been developed for the treatment of patients with thyroid carcinoma-related diseases.

This investigation deals with the preparation of a PEGylated liposomal formulation containing ATRA, its physico-chemical characterization, and the evaluation of its anticancer activity on different human thyroid cancer cell lines (PTC-1, B-CPAP and FRO) as compared to the free form. The rationale of this approach is related to the differentiating activity of ATRA, which could act as an ideal anticancer agent against anaplastic thyroid carcinoma.

2. Experimental section

2.1. Chemicals

The phospholipids used for the preparation of liposomes, dipalmitoyl-sn-*glycero*-3-phosphatidylcholine monohydrate (DPPC), the cholesterol (Chol), the N-(fluorescein-5-tiocarbamoyl)-1,2,dihexadecanoyl-sn-*glycero*-3-phosphoethanolamine triethylammonium salt (fluorescein-DHPE) and All-*trans*-Retinoic Acid (ATRA) were purchased from Sigma Aldrich (Milan, Italy). N-(carbonyl-methoxipolyethylene glycol-2000)-1,2-distearoyl-sn-*glycero*-3-phosphoethanolamine (DSPE-mPEG2000) was provided by Avanti Polar Lipids (Spectra 2000, Rome, Italy). RPMI-1640 and D-MEM culture media, fetal bovine serum (FBS), trypsin-EDTA (1 ×) solution and penicillin-streptomycin solution were obtained from GIBCO (Life Technologies, San Giuliano Milanese, Milan, Italy). Human thyroid tumor cell lines (FRO, PTC-1, B-CPAP) were provided by the Istituto Zooprofilattico of Modena and Reggio Emilia. All other materials and solvents used in this investigation were of analytical grade (Carlo Erba, Milan, Italy).

2.2. Laboratory precautions

Being a drug particularly sensitive to light and oxygen, the retinoic acid was carefully handled in darkrooms with low oxygen contents, and particular care was always taken to keep the formulations in environments protected from light.

2.3. Liposome preparation

The liposomes were prepared as previously described [27]. Briefly, a lipid mixture (20 mg) made up of DPPC:Chol:DSPE-mPEG2000 (6:3:1 molar ratio) was dissolved in 1 ml of chloroform/methanol (3:1 v/v). ATRA (20:1 w/w lipids:ATRA), due to its high lipophilicity, was added to the phospholipid mixture before dissolution in the organic solvents. The solvent was removed using a rotavapor Büchi R-210 (Flawil, Switzerland) and overnight storage at room temperature (Büchi T51 glass drying oven connected to a vacuum pump) allowed the formation of a thin film layer on the inner walls of the pyrex glass tubes used. The films were hydrated with 1 ml of bidistilled water or NaCl (0.9% w/v) aqueous solution and submitted to three alternate cycles (3 min each) of warming at 58 °C in a thermostatic water bath and vigorous mixing by vortexing at 11 × g. The multilamellar liposomes thus obtained were kept at 57–60 °C for 3 h to anneal the bilayer structure.

To reduce both liposome lamellarity and mean size, multilamellar liposomes were extruded by means of a Lipex extruder (Vancouver, Canada) through polycarbonate membrane filters (Nucleopore® Polycarbonate). The liposomes were initially extruded using two 400 nm polycarbonate filters of (10 cycles), then by two 200 nm polycarbonate filters of (10 cycles). The working pressure was 450 and 880 kPa for the 400 nm and 200 nm filters, respectively. The extrusion procedure led to the formation of unilamellar liposomes [28]. Fluorescent liposomes were prepared by co-dissolving fluorescein-DHPE (0.1%) in the lipid mixture. Sterile liposomes were prepared by means of suitable filtration through a laminar vertical flow cabinet (Steril VBH Compact mod. 72C2).

2.4. Characterization of liposomes

Mean size, size distribution and z-potential were evaluated following a 1:50 dilution of the samples by a Zetasizer Nano ZS (Malvern Instruments Ltd., Worchestershire, United Kingdom), which is a dynamic light-scattering spectrophotometer with an applied third-order cumulant fitting correlation function. A 4.5 mW laser diode operating at 670 nm was used as a light source for size analysis and back-scattered photons were detected at 173°. The real and imaginary refractive indexes were set at 1.59 and 0.0, respectively. The medium refractive index (1.330), medium viscosity (1.0 mPa × s), and dielectric constant (80.4) were set before the experiments. Quartz cuvettes were used for the analysis. The stability of the carriers was determined by using a Turbiscan Lab® Expert [29]. Briefly, the samples were placed into a cylindrical glass tube and measurements were performed for 3 h. The photon which was transmitted (T) and backscattered (BS) through the whole 6 mm height of the sample was recorded. Analysis was carried out at the temperature of 24 ± 1 °C. The analyses were performed using TurbiSoft software (Formulaction, L'Union, France) and the obtained data were used to evaluate the kinetic stability of the formulations.

2.5. Serum liposome stability

The influence of serum on the physico-chemical properties of the extruded liposomes was investigated through the incubation of the colloids in 60% FBS (diluted in PBS) at 37 °C. The liposomes (200 μl) were added to 1 ml of FBS solution [30]. Time course studies for up to 6 days were performed at constant stirring. For-

Please cite this article in press as: M.C. Cristiano, et al., Anticancer activity of all-*trans* retinoic acid-loaded liposomes on human thyroid carcinoma cells, Colloids Surf. B: Biointerfaces (2016), http://dx.doi.org/10.1016/j.colsurfb.2016.10.052