Anticancer activity of 9-*cis*-retinoic acid encapsulated in PEG-coated PLGA-nanoparticles

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The use of retinoids in antitumor therapy represents a very promising approach for the treatment of undifferentiated tumors, due to their ability to promote cell differentiation. Among these compounds, 9-cis-retinoic acid (9-cis-RA) is the most active. The therapeutic use of 9-cis-RA is made difficult by the occurrence of several side effects in addition to its photo-degradation following exposition to light or atmospheric oxygen. In this investigation, 9-cis-RA was encapsulated in PEG-coated PLGA nanoparticles to improve both its stability and effectiveness. PEG-coated PLGA nanoparticles showed a mean size of ~220 nm, a polydispersity index of ~0.1 and a zeta potential of -12 mV. These parameters were not significantly influenced by the presence of the 9-cis-RA. Encapsulation provided noticeable protection of the 9-cis-RA. In vitro experiments showed that the encapsulated form is more active than the free drug in inducing cell differentiation.

Key words: PLGA nanoparticles – 9-cis-retinoic acid – Drug photo-stabilization – In vitro activity – ARO cells – PTC-1 cells – Cell differentiation.

The retinoids are a group of compounds that are chemically related to Vitamin A (Retinol). Retinoids are lipophilic compounds formed by four isoprenoid units joined in a head-to-tail manner. Their structure consists of a cyclic group and a polar group separated by a ramified polyen chain. This structure, characterized by an alternated sequence of five double carbon-carbon bonds, is responsible for the ease of destabilization following light exposition which leads to photodegradation [1, 2].

Natural retinoids play an important role in vision, bone growth, cell division and cell differentiation [3] and they also improve the functionality of the immune system by preventing both cancer and viral/bacterial infections, thanks to their ability to increase the production and the activity of white blood cells and lymphocytes [4, 5].

These features and the ability of retinoids to inhibit or at least to delay the transformation of a normal cell into a cancer cell, to promote its differentiation, to inhibit the growth and the diffusion of cancer cells and to promote their death by apoptosis, make them a useful tool in the treatment of many types of cancerous diseases [6, 7].

Several actions seem to be involved in the anticancer activity of these compounds: i) inhibition of cellular proliferation through various mechanisms, including induction of apoptosis [8-10], ii) blockage of the tumor cell cycle in the G0/G1 phase [11], iii) inhibition of activator protein-1 (AP-1) and, consequently, of chemical carcinogenesis [12], and iv) reduction of matrilysin expression. This metalloproteinase plays a crucial role in the invasion and metastatic diffusion of some cancers and hence the reduction of the invasive capacity of some tumors [13].

Three geometric isomers of retinoic acid have been recognized to be biologically active as anticancer agents, i.e. all-*trans*-retinoic acid or tretinoin (ATRA), 9-*cis* retinoic acid or alitretinoin (9-*cis*-RA) and 13-*cis* retinoic acid or isotretinoin (13-*cis*-RA). Amongst these three, 9-*cis*-RA is the most active in inducing morphological differentiation and inhibiting proliferation [14]. The biological activity of these isomers, (besides some of the toxicological effects of these compounds) depends on their interaction with two kinds of nuclear receptors: the retinoic acid receptor (RAR) and the retinoid X receptor (RXR), which act as ligand-activated transcription factors [15]. The prolonged administration of retinoids can determine the appearance of some side effects, i.e. anorexia, hepatosplenomegaly, papilloedema, hair loss and potential teratogenicity. A way to modulate the biopharmaceutical properties of the active compounds after i.v. administration is based on their encapsulation in colloidal nanodevices with the aim of increasing their pharmacological action, protecting them from a rapid metabolism and accumulating in specific areas [16-18]. Polymeric nanoparticles, made up of PLGA, allow the improvement of the pharmacokinetic and pharmacodynamic features of different drugs, avoiding the appearance of any side effects thanks to the total biocompatibility of the polymer [19, 20]. Moreover, the presence of the polyethylene glycol (PEG) on the colloidal surface favors a prolonged blood circulation time of the carrier and consequently of the encapsulated compound, as a consequence of the reduced opsonization phenomena. In particular, this feature allows the passive targeting of the drug-loaded nanoparticles in solid tumors by means of the enhanced permeation and retention (EPR) effect [21, 22].

The aim of this investigation was the encapsulation of 9-*cis*-RA in PEG-coated PLGA (*Pc*-PLGA) nanoparticles in order to protect the drug from photo-chemical degradation and to improve its anticancer activity.

I. MATERIALS AND METHODS
1. Materials

The poly-lactic-co-glycolic acid copolymer (PLGA) (Resomer RG 752H, Mw 4000-15000) was purchased by Boehringer Ingelheim Pharma GmbH & Co. KG (Germany), Tween 80 was provided from Acef S.p.a. (Fiorenzuola D'Arda, Piacenza, Italy) and acetone was obtained from Carlo Erba (Milano, Italy). N-(carbonyl-methoxypolyethylene glycol-2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE-MPEG 2000) was purchased from Genzyme (Suffolk, United Kingdom). 9-*cis*-RA was purchased from Sigma Chemical Company (St. Louis, United States). Thyroid cancer cell lines from anaplastic carcinoma and those from papillary carcinoma were supplied by G. Juillard, UCLA. For the *in vitro* studies, RPMI and DMEM (Dulbecco's modified eagle's medium) culture media, enriched with Glutamax I, tripsin/EDTA, penicillin/streptomycin solution and fetal bovine serum (FBS) were obtained from GIBCO (Invitrogen Corporation, United

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Kingdom). All materials and solvents used throughout this investigation were of analytical grade (Carlo Erba, Milano, Italy).

2. Preparation of 9-*cis*-RA-loaded PEG-coated PLGA nanoparticles

The nanoparticles were prepared following the nanoprecipitation method of the pre-formed polymer in a micellar aqueous solution [23]. The polymer Resomer RG 752H (30 mg) was dissolved in 4 mL of acetone. This organic solution was added to an aqueous solution (10 mL) of Tween 80 (3 % w/v) under moderate stirring using a magnetic anchor. The suspension was homogenized with ultraturrax (IKA T25, Werke, Germany) at 24000 rpm for 1 min and then it was mechanically stirred at 600 rpm for 3 h with the aim of allowing the solvent to evaporate completely. In order to obtain a PEGylated system, DSPE-mPEG2000 (0.05 % w/v) was added to the organic phase during the preparation process. When 9-*cis*-RA-loaded *Pc*-PLGA nanoparticles were needed, different amounts of drug were dissolved in the organic phase due to its highly lipophilic character (*Figure 1*). Considering the sensitivity of the drug to light and atmospheric oxygen, the 9-*cis*-RA was carefully handled in a darkroom within a hermetically sealed box in an oxygen-free atmosphere. The nanoparticles were then purified of un-entrapped drug and other unstructured compounds used during the preparation by ultracentrifugation at 28000 rpm for 60 min at 4 °C. The centrifuge was a Beckman Avanti 30 (Fullerton, United States), equipped with a fixed angle rotor F12. The pellet of 9-*cis*-RA-loaded *Pc*-PLGA nanoparticles was washed twice and resuspended in saline solution for future investigations.

3. Physicochemical characterization

Photon correlation spectroscopy was used to evaluate mean sizes, zeta potential and polydispersity index of uncoated and *Pc*-PLGA nanoparticles in the presence and the absence of 9-*cis*-RA. A Zetasizer Nano ZS (Malvern Instruments Ltd., Worchestershire, United Kingdom) was used for these experiments. A 4.5 mW laser diode operating at 670 nm was used as a light source for size analysis and the back-scattered photons were detected at 173 °. A third-order cumulant fitting correlation function was applied. 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 the dielectric constant (80.4) were set before the experiments. Quartz cuvettes were used for the analysis. The instrument was calibrated with a standard sample of monodisperse polystyrene latex (126 nm).

The Zetasizer Nano ZS was also used for Z-potential determination by applying a Smoluchowsky constant F (Ka) of 1.5 to calculate the zeta-potential value as a function of the electrophoretic mobility of the nanoparticles. The various measurements were carried out in triplicate on three different batches (10 determinations for each batch). Results were expressed as the mean of three different experiments ± standard deviation.

4. Loading capacity evaluation

The amount of 9-*cis*-RA entrapped within the *Pc*-PLGA nanoparticles was determined spectrophotometrically following the destruction of the colloidal carriers. Briefly, 9-*cis*-RA-loaded *Pc*-PLGA nanoparticle formulations (3 mL) were centrifuged twice at 50000×g for 1 h at 4 °C by a Beckman Coulter Allegra 64R centrifuge in order to discharge the supernatant and solubilize the pellet in 1 mL of ethanol. The 9-*cis*-RA was determined spectrophotometrically (PerkinElmer Lambda 25 UV-Vis spectrophotometer) at a λ_{max} of 340 nm using a PerkinElmer UV WinLabTM 2.8 acquisition software (Perkin-Elmer GmbH Uberlingen, Germany). An empty nanoparticle formulation was used as blank. A calibration curve was carried out to determine the amount of the drug by reporting the known concentrations of the drug as a function of the respective absorbances. The curve equation was:
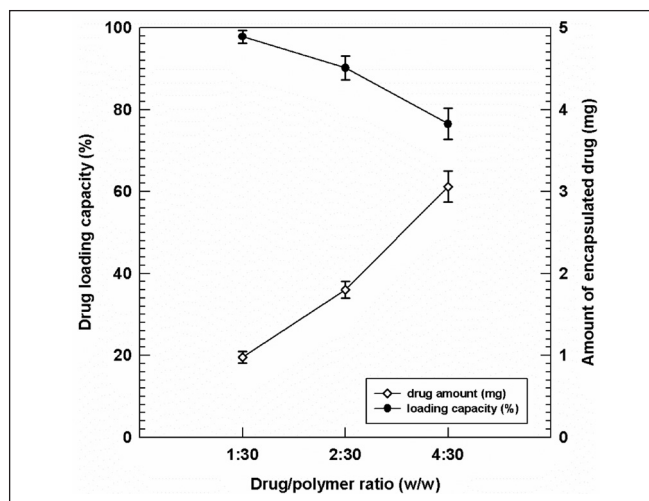
Figure 1 - Variation of the 9-*cis*-RA loading capacity as a function of drug/polymer ratio. The experiments were carried out at room temperature. Each value is the mean of three different experiments ± standard deviation. Each value showed a coefficient of variation (standard deviation/mean) < 0.1.

$$y = 0.3671x - 0.3265 \qquad \textbf{Eq. 1}$$

where x was the concentration and y the absorbance. The coefficient of linear regression was $r^2 = 0.9965$.

5. Stability investigation

An ethanol solution of 15.0 µg/mL of 9-*cis*-RA, in a 1 × 1 cm quartz cuvette perfectly stopped, was irradiated with a Xenon lamp according to the ICH Guideline for photo-stability testing [24] and the UV spectra were recorded by means of the aforesaid spectrophotometer at pre-determined times of 0, 30, 60, 120, 180, and 240 min. In particular, samples were irradiated at wavelength ranging from 300 to 800 nm, by means of a glass filter, according to the ID65 standard of ICH rules. Irradiance power was set to 250 W/m², corresponding to a light dose of 21 kJmin^{-1}m^{-2}, at the constant temperature of 25 °C. Likewise, 9-*cis*-RA-loaded *Pc*-PLGA nanoparticles were subjected to the same irradiation process and then a rate of the nanoparticle formulation containing 15.0 µg of the active compound was dissolved in 1 mL of ethanol and its UV spectrum recorded. In this case, an empty nanoparticle formulation was used as the blank.

6. Release experiments

The release rate of the 9-*cis*-RA from the *Pc*-PLGA nanoparticles was evaluated by using the dynamic Franz-type diffusion cells. Each cell consists of a donor and a receptor chamber characterized by a 0.75 cm² diffusion surface area and a nominal receptor volume of 4.75 mL, separated by a cellulose acetate membrane with a molecular cut off of 10,000 Da, which was previously hydrated in water at room temperature for 60 min. The chambers and diffusion membranes were firmly tightened using suitable clips and the system was allowed to equilibrate for 6 h. Samples of nanoparticle suspension (200 µL) were placed into the donor chamber. The receptor fluid was made up of a water/ethanol (70:30 v:v) mixture and was maintained under constant stirring (600 rpm) to avoid the formation of layers of stagnant fluid near the surface of the membrane. The receptor fluid ensured sink conditions throughout the release experiments. At intervals of 1 h for a duration of 24 h, 1 mL of receptor fluid was withdrawn from each sample using a peristaltic pump and a fraction collector (Gilson FC 204, France). The withdrawn volume was replaced with an equal volume of fresh receptor fluid. Samples of receptor fluid were analyzed by UV spectrophotometry at the 9-*cis*-RA λ_{max} 340 nm. The release was calculated according to the following equation: