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Nutlin-3 loaded nanocarriers: Preparation, characterization and *in vitro* antineoplastic effect against primary effusion lymphoma

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ABSTRACT

In this investigation, Nutlin-3 (Nut3), a novel antitumor drug with low water solubility (<0.1 mg/L at 25 °C), was loaded into liposomes (Lipo-Nut3), polymeric nanoparticles (NPs-Nut3) and nanoparticles engineered with an antibody direct against Syndecan-1/CD 138 (Syn-NPs-Nut3) to obtain carriers targeted to PEL (primary effusion lymphoma). The physicochemical properties of these carriers were determined. Atomic force microscopy showed that all the particles were well formed and spherical in shape. The presence of the antibody on surface led to a significant increase of mean diameter (280 ± 63 nm), PDI (0.3) and the shift of zeta potential towards neutrality (−1 mV). The entrapment efficiency of Lipo-Nut3, NPs-Nut3 and Syn-NPs-Nut3 was 30, 52 and 29%, and drug loading was 1.4, 4.5 and 2.6%, respectively. By performing cytofluorimetric analyses and bromodeoxyuridine (BrdU) assay, the efficacy of nanocarriers to deliver the antineoplastic drug into a PEL cell line namely BCBL-1 (immortalized body cavity B-cell lymphoma) was investigated. Two days after the treatment with 20 μM of Syn-NPs-Nut3, the cell density decreased at about 60% while the cell viability decreased at 56% only 5 days after transfection, when compared with untreated cells. A cell cycle arrest was observed with a significant decrease of cells in S-phase and increasing of apoptotic cell, if compared with untreated control. These results confirms the potential of nanocarriers approaches to deliver antitumor drug with unfavorable chemico-physical properties. Moreover, this study strongly suggests that Syn-NPs-Nut3 can be a valuable drug carrier system for the treatment of PEL lymphoma.

1. Introduction

Nutlin-3 (Nut3) is an anticancer small-drug molecule belonging to the selective antagonist of MDM2 recently identified from the class of cis-imidazoline compounds (Vassilev et al., 2004).

MDM2, the main negative regulator of p53, the "guardian of the genome", is aberrantly expressed in many tumors (Bond et al., 2005). Nut3 though the inhibition of p53-MDM2 complex induces the reactivation of the p53 pathway leading to cell cycle arrest, growth inhibition, and apoptosis of cancer cells.

Although originally tested in solid tumors, a growing number of studies have recently described the antineoplastic activities of Nut3 in hematological malignances characterized by wild-type (wt) p53. Nut3 has been tested on chronic lymphocytic leukemia (Kojima et al., 2006), Hodgkin's lymphomas (Drakos et al., 2007) and most recently primary effusion lymphomas (PEL) (Sarek et al., 2007; Petre et al., 2006), which is the focus of our study.

PEL is a B cell non-Hodgkin lymphoma involving the serous cavities invariably associated with Human HerpesVirus-8 (HHV-8). Generally, PEL therapy is ineffective in the majority of patients, who are invariably characterized by a poor prognosis. Being the majority of PEL cases characterized by wt p53, Nut3 might be considered as a possible alternative respect to conventional chemotherapy. Moreover it is appreciable its low risk to induce drug resistance and the absence of genotoxic damage (Sarek et al., 2007).

Unfortunately, Nut3 is sparingly soluble in aqueous buffer causing difficulty during administration (frequently *per os*) and poor bioavailability. Nanotechnologies offer a very powerful tool to improve the pharmacokinetic profile of drugs (Farokhzad and Langer, 2009). In this contest, liposomes and nanoparticles can be applied because they are able to encapsulate, stabilize and increase the drug bioavailability (Fahr and Liu, 2007). Moreover, when

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opportunely modified on their surface, liposomes and nanoparticles demonstrated the capability to target the drug to the site of action (Wicki et al., 2015).

In a previous paper, we demonstrated that nanoparticles engineered with Rituximab and loaded with Nut3 showed promising therapeutic antitumor activity both *in vitro* and *in vivo* in models of B-cell malignances (Voltan et al., 2013a,b). This work aims to optimize the delivery of Nut3 in PEL cells for applying the drug also in PEL therapy. After comparison with the literature data, we proposed DOPC/Chol liposomes (Lipo-Nut3) and polylactide-co-glycolide (PLGA) nanoparticles (NPs-Nut3), opportunely formulated to improve the encapsulation efficiency and the transfectability in PEL target cells.

2. Materials and methods

2.1. Materials

Cholesterol (Chol) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were obtained from Sigma Chemical Co. (St. Louis Mo, USA). Nutlin-3 (Nut3) was purchased from Cayman Chemical (Neratovice, Czech Republic). Poly (d,L-lactide-co-glycolide) acid (PLGA RG-503H 50:50, inherent viscosity in 0.1% (w/v) $CHCl_3$ at 25 °C = 0.38 dL/g) was used as received from the manufacturer (Boehringer-Ingelheim, Ingelheim am Rhein, Germany). According to the experimental titration results of the carboxylic end of the polymers (4.94 mg KOH/g polymer) the molecular weight of RG-503H was calculated to be 11,000 Da. The human monoclonal antibody against CD-138/Syndecan-1 (anti-Syndecan-1), RPMI 1640, culture media, fetal calf serum (FCS) and phosphate buffered saline pH 7.4 were purchased from Invitrogen (Milan, Italy). Sephadex G-150, acetonitrile (HPLC grade), and analytical grade of methanol and chloroform were purchased from Carlo Erba (Milan, Italy). High pure water was prepared by using Milli-Q plus filtration system (Millipore, Molsheim, France). All the other chemicals were obtained commercially and used without further purification.

2.2. Preparation of Nut3 loaded liposomes

The Nut3 loaded liposomes (Lipo-Nut3) were prepared by a thin layer evaporation method followed by extrusion through a 200 nm polycarbonate filter (Liposofast Basic; Avestin, Ottawa, Canada) (Ruozi et al., 2010). The lipidic mixture composed of DOPC/Chol (9:1 molar ratio, 10 mg) was dissolved in chloroform (4 mL). Then 1 mL of a chloroformic solution of Nut3 (0.5 mg) was added to the lipidic solution. The solvent was removed by rotary evaporation (B-480 Büchi, Büchs, Switzerland) for 1 h under vacuum (10 mbar) at 20 ± 1 °C. The dried lipidic film was further vacuum dried for 3 h (0.15 mbar) at the same temperature and then hydrated with 5 mL of water preheated to 37 °C. The hydrated preparation was alternatively vortexed for 3 min (Zx3, Velp Scientifica, Usmate, Italy) and warmed in a water bath at 40 ± 2 °C for 3 min, for three cycles. The formulations were extruded using a 200 nm polycarbonate filter (19 cycles). The unloaded free Nut3 was removed from the preparation by gel filtration chromatography (Sepharose® CL-2B gel, column 25 cm × 2 cm) and eluting in water (0.7 mL/min). Then, the Lipo-Nut3 were quickly frozen with liquid nitrogen and freeze-dried (−60 °C, 1×10^{-3} mm/Hg; LyoLab 3000, Heto-Holten, Allerod, Denmark) for 24 h using D-(+)-trehalose (Fluka–Sigma–Aldrich) as cryoprotectant at 1:2 w/w lipid/trehalose ratio. Empty liposomes (Lipo) were prepared similarly but without adding the drug. All the freeze dried liposome preparations were stored at 4 °C and re-suspended in water before the use. Each formulation was prepared in triplicate.

2.3. Preparation of Nut3 loaded nanoparticles

Nut3 loaded nanoparticles (NPs-Nut3) were obtained in accordance with the nanoprecipitation procedure (Tosi et al., 2007). Briefly, the PLGA RG-503H (50 mg) and Nut3 (5 mg) were solubilized in acetone (4 mL). The organic solution was then added dropwise into a 1% (w/v) PVA aqueous solution (6 mL). The solution was then stirred at r.t. for 15 min and the organic solvent was removed at 30 °C under reduced pressure (10 mm Hg). Nanoparticle preparations were then purified by centrifugation (17,000 rpm for 10 min, 4 °C; Sorvall RC28S, Dupont, Brussels, Belgium), washed and re-suspended in water.

Anti-syndecan-1 engineered nanoparticles (Syn-NPs-Nut3) were prepared starting from drug loaded PLGA-nanoparticles (NPs-Nut3) applying the methodology for antibody-surface engineering of nanoparticles previously described (Bondioli et al., 2010; Nobs et al., 2003). Briefly, 25 mg of each preparation were suspended in 2-(*N*-morpholino) ethanesulfonic acid (MES, Sigma–Aldrich) buffer and added with 25 mg of *N*-hydroxysuccinimide (NHS, Sigma–Aldrich) and 75 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC; Sigma–Aldrich) to activate the carboxylic groups of the polymer. After 1 h the activated nanoparticles were collected by ultracentrifugation at 17,000 rpm for 10 min at 4 °C and the excess of reagents were removed. NPs were re-suspended in PBS (pH 7.4) to have 10 mg of NPs/mL and stirred at r.t. for 1 h with the designated volume of anti-Syndecan monoclonal antibody (500 μL of a 100 μg/mL stock solution) in order to obtain Syn-NPs-Nut3. After the reaction, the suspension of the Syn-NPs-Nut3 was collected by centrifugation and further washed twice by distilled water. Syn-NPs-Nut3 were then freeze-dried (−60 °C, 1×10^{-3} mm/Hg; LyoLab 3000, Heto-Holten) at r.t. by using D-(+)-trehalose (Fluka–Sigma–Aldrich) as cryo-protectant at 1:1 w/w polymer/trehalose ratio. Empty NPs (NPs) and empty antibody conjugated nanoparticles (Syn-NPs) were also prepared similarly but without adding the drug to the preparation. All the nanoparticles preparations were stored at 4 °C and re-suspended in water before the use. Each formulation was prepared in triplicate.

2.4. Characterization of the nanocarriers

The mean diameter (Z-average), the size distribution [expressed as D(50) and D(90)] and the polydispersity index (PDI) of the samples were determined at 25 °C by photon correlation spectroscopy (PCS) using a Zetasizer Nano ZS (Malvern, Malvern, UK; Laser 4 mW He–Ne, 633 nm, laser attenuator automatic, transmission 100–0.0003%, detector avalanche photodiode, Q.E. >50% at 633 nm). For each formulation, the mean diameter and PDI were calculated as the mean of three replicates of three different batches (9 measurements). The zeta potential (ζ-potential) was measured using the same equipment with a combination of laser Doppler velocimetry and phase analysis light scattering (PALS). Data of ζ-potential were collected as the average of 10 measurements.

The AFM observations were performed with an atomic force microscope (Park Instruments, Sunnyvale, CA, USA) at about 20 °C operating in air and in non-contact mode using a commercial silicon tip-cantilever (high resolution noncontact "GOLDEN" Silicon Cantilevers NSG-11, NT-MDT, tip diameter 5–10 nm; Zelenograd, Moscow, Russia) with stiffness about 40 Nm^{-1} and a resonance frequency around 150 kHz. After the purification, the sample was dispersed in distilled water before to be applied on a freshly cleaved mica disk (1 cm × 1 cm); two minutes after the deposition, the water excess was removed using a blotting paper. The AFM images were obtained with a scan rate 1 Hz. Two kinds of images are obtained: the first one is a topographical image and the