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Short communication

pH-sensitive niosomal ATRA delivery as a promising approach to inhibit Pin1 in High-Grade Serous Ovarian Cancer

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Introduction

High-grade serous epithelial ovarian cancer (HGSOC) is a deadly disease, which accounts for >150.000 deaths each year worldwide. For decades, treatment strategies for HGSOC have shown little improvement in overall survival and the use of cytoreductive surgery followed by platinum-based chemotherapy remains the first-line treatment. Although most patients respond to platinum based therapy, the majority relapse and die from the disease.

A fundamental mechanism in controlling key proteins in these pathways is the phosphorylation of the proline (Pro)-Ser/Thr motifs, which are controlled by the Peptidyl-prolyl cis-trans isomerase NIMAinteracting 1 (Pin1), a unique Peptidyl-prolyl isomerase (PPIase). Pin1 accelerates the conversion of cis and trans isomers, which is slowed down by phosphorylation and the net result is the activation of oncogenes and inactivation of tumor suppressor genes in cancer cells; therefore, its inhibition represents an exciting therapeutic target for the treatment of HGSOCs (Russo et al., 2018). All trans retinoic acid (ATRA) inhibits and degrades Pin1 selectively in cancer cells by directly binding to the substrate phosphate- and proline-binding pockets in the Pin1 active site (Wei et al., 2015). Given that Pin1 is overexpressed in a wide range of tumors and it sustains several oncogenic pathways, these findings fostered the application of ATRA in the treatment of a great variety of solid tumors. Despite the wide spectrum of functions shown by ATRA, its therapeutic use is severely limited due to the development in the patients of 'acute retinoid resistance' (Giuli et al., 2020). In addition, ATRA displays low aqueous solubility which does not allow parenteral administration, its susceptibility to light, heat and oxidants and reduced half-life in plasma due to the metabolism regulation of CYP-450 in the liver, hindering its biomedical usage. To overcome ATRA issues related, in this work it has been loaded into niosomes, liposomes-like structures are surfactant based self-assembling vesicular nanocarriers, already used for anticancer therapy. Typically, cancer cells are able to generate energy by glycolysis in hypoxic environment, then the extracellular environment of tumor is characterized by a lower pH (6.5–6.8) compared to normal tissues (pH 7.4). In order to take advantage from this effect, in this work the pH-sensitiveness of vesicular structures to improve ATRA release in a pH-controlled manner has been evaluated.

Materials and methods

ATRA loaded niosomes composed of 15 mM of Tween 20 or Tween 21, cholesterol (15 mM) and ATRA (0.5 mg/ml) are obtained by 'film' method and purified using a size exclusion chromatography on Sephadex G25. All samples are analyzed using Zetasizer Nano ZS 90, Malvern, UK to assure vesicle formation and characterize them in terms of Z-Average hydrodynamic diameter, ζ-potential and polydispersity index (PDI) to check the sample homogeneity (Table 1). In parallel, the entrapment efficiency and release studies of ATRA from the niosomal vesicles were measured, using a UV–visible spectroscopy to quantify the final ATRA concentration after purification, and to check the ability of vesicle to release ATRA at different pH and in presence of human serum (HS) in 24h.

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Table 1. Dynamic Light Scattering results

Sample	Size (nm)	ζ-potential	PDI	[ATRA] (mg/ml)
NioTW20	281	-40.6	0.4	-
NioTW20-A	251.4	-30.5	0.3	0.08
NioTW21	175.1	-43.4	0.3	-
NioTW21-A	190.7	-40.0	0.4	0.06

The pH-sensitivity of nanosystems was investigated evaluating variations of size, PDI, ζ-potential, stability over time, microviscosity, polarity at pH 7.4 and at pH 5.5 (Table 2). Fluorescence analyses were carried out by Perkin-Elmer LS50B spectrofluorometer on HPTS-loaded and on calcein-loaded vesicles prepared in Hepes buffer (pH 7.4) or acetate buffer (pH 5.5).

Table 2. Polarity and Microviscosity results

Sample	Buffer	Polarity (I_1/I_3)	Microviscosity (I_E/I_3)
NioTW20	Hepes	1.44	0.28
	Acetate	1.57	0.12
NioTW20-A	Hepes	1.60	0.15
	Acetate	1.70	0.07
NioTW21	Hepes	1.28	0.34
	Acetate	1.40	0.26
NioTW21-A	Hepes	1.59	0.19
	Acetate	1.65	0.07

The influence of HS different concentrations on the in vitro stability and pH-sensitivity of ATRA Calcein-loaded at 37°C has been evaluated. The vesicles were incubated at the two different pH in absence and in presence of 50% of HS. Calcein leakage was evaluated after 3h of incubation fluorimetrically. Stability studies over time of empty and loaded niosomes were developed at 4°C and room temperature for 30 days in Hepes buffer by measuring hydrodynamic diameter and ζ-potential.

Cell viability assay was carried out on OVCAR-3, OVSAHO and Kuramochi cells treated with empty niosomes. Western Blot analysis were also performed to show Pin1 endogenous protein levels of the above-mentioned cell lines treated with ATRA loaded niosomes.

Results and discussion

The pH-sensitivity of nanosystems was demonstrated evaluating size and ζ-potential values of samples at different pH. In particular the structures at pH 7.4 are about 175-281 nm, with a ζ-potential (-30/-43mV) which allows to predict a good stability of the suspensions. At pH 5.5 the samples have larger dimensions about 500-700 nm. The destabilization of niosomal structure at more acidic pH was also confirmed through the use of fluorescent probe Pyrene, that evidenced a reduction in microviscosity and an increase in polarity of the vesicular bilayer (Table 2). The amount of ATRA released over time in Hepes buffer (pH 7.4), in Acetate buffer (pH 5.5) and in presence of HS has been evaluated at 37°C for 24h. In particular, the ATRA release increased in the presence of Acetate buffer and HS: after 2h the ATRA release is about 50% and after 24h is about 80%. The experiments confirmed the nanocarriers pH-sensitivity. The effect of HS on vesicle stability did not modify vesicle stability.

Moreover, *in vitro* biological studies suggest that empty niosomes were not toxic for cells (data not shown). Interestingly, ATRA loaded niosomes significantly decreased Pin1 level with respect to ATRA free (Figure 1).

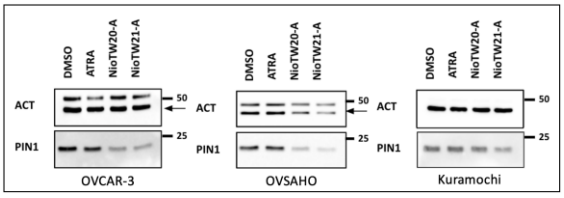

Figure 1. Example figure to show Western Blot results

Conclusion

Encapsulation of ATRA in niosomal vesicles could represent a promising approach to improve the aqueous solubility and bioavailability. Furthermore, the choice of these non-ionic surfactant based niosomes allow us to study also the pH-sensitiveness of vesicles which allow them to easily penetrate into tumor area. Furthermore, encapsulation of ATRA exhibited a down-regulation of Pin1 whereas ATRA free had almost no effect.

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