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In vitro and ex vivo delivery of tailored siRNA-nanoliposomes for E2F1 silencing as a potential therapy for colorectal cancer

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DOTAP- *N*-[1-(2,3-Dioleoyloxy)Propyl]-*N*,*N*,*N*-trimethylammonium Chloride (PubChem CID: 123922)
Cholesterol (PubChem CID: 5997)
3-sn-Phosphatidylcholine (PubChem CID: 16213884)
MTT -3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (PubChem CID: 64966)

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ABSTRACT

Tailored developed nanoliposomes loaded with a siRNA against the transcription factor E2F1 (siE2F1), were produced and delivered to human colorectal adenocarcinoma cell lines and to intestinal human biopsies. siE2F1 loaded nanoliposomes were produced through a dedicated ultrasound assisted technique producing particles with about 40 nm size (Small Unilamellar Vesicles, SUVs) and 100% siRNA encapsulation efficiency. Compared to other production methods, the one proposed here can easily produce particles in the nanometric scale by suitable ultrasonic duty cycle treatments. Furthermore, SUVs have a high degree of size homogeneity, a relevant feature for uniform delivery behaviour.

siE2F1-loaded SUVs demonstrated a very low cytotoxicity in cells when compared to a commercial transfection agent. Moreover, SUVs loaded with siE2F1 were effective in the down regulation of the target in cultured colon carcinoma cells and in the consequent reduction of cell growth. Finally, a remarkable uptake and target silencing efficiencies were observed in cultured human biopsy of colonic mucosa. In conclusion, whereas further studies in more complex models are required, the siE2F1-SUVs generated have the potential to contribute to the development of novel effective inflammatory bowel diseases-associated colorectal cancer therapies for a future personalized medicine.

1. Introduction

The discovery of RNA interference (RNAi) mechanism and its effector molecule, the short interfering RNAs (siRNAs), has led to an increased interest in the development of innovative therapies to target pathologic RNAs. However, while the selection of effective siRNA sequences has progressed significantly, the scientific community is still busily focused on the development of suitable drug delivery systems (DDSs). Indeed, due to the low stability in physiological fluids, low membrane-permeability and the short half-life in the circulatory system, siRNAs require to be encapsulated in suitable carriers. In general, the main features of an ideal drug delivery system are biodegradability, low intrinsic toxicity, accumulation in pathological areas, high drug loading, prolonged half-life in the bloodstream, low cost, easiness of preparation and reduced size (Bochicchio et al., 2017). Other desirable features are the versatility and the easiness of carriers modification in terms of size, charge, composition and the possibility to be equipped with cell-targeting molecules (Barba et al., 2015; Bochicchio et al., 2014). Because of the possibility to adhere to these requirements, liposomes are considered ideal candidates for siRNAs incorporation and delivery. However, not all liposomes are suitable as,

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Nomenclature

CHOL	Cholesterol
CD	Crohn's disease
DAPI	4′6-Diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle's medium
DOTAP	*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride
DDSs	Drug delivery systems
dsDNA	Double stranded DNA
EE	Encapsulation efficiency
E2F1	Transcription factor
EPR	Enhanced permeability and retention
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HT29	Human-colon carcinoma cell line
IBDs	Inflammatory bowel diseases
LVs	Large vesicles
LoVo109	Human colon-carcinoma cell line
MLVs	Multilamellar vesicles
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
PC	L-α-phosphatidylcholine
PBS	Phosphate buffer solution
PDI	Polidispersity index
RNAi	RNA interference
RPMI	Roswell Park Memorial Institute Medium
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
siRNAs	Short interfering RNAs
siE2F1	siRNA against the transcription factor E2F1
SUVs	Small unilamellar vesicles
TBS	Tris/borate/EDTA buffer
UC	Colitis ulcerosa

depending on their lipid composition and size, they can induce different levels of un-specific cell toxicity, can have low delivery effectiveness and/or have poor siRNA loading capacity. In this regard, liposomes in the nanometric range, known as Small Unilamellar Vesicles (SUVs), are particularly attracting. Indeed, due to the Enhanced Permeability and Retention (EPR) effect (Bregoli et al., 2016), nanoliposomes can permeate through membrane fenestrations of diseased blood vessels penetrating into the tumour tissue (Kibria et al., 2016). Moreover, positively charged liposomes are preferable to enhance the interaction with the negatively charged siRNAs and the negatively charged cell plasmatic membrane, thus improving siRNA encapsulation and eventually the uptake into target cells (Ibraheem et al., 2014; Kim et al., 2010).

For all the above stated reasons, in the last few years liposomes have been increasingly investigated for the development of novel strategies to deliver therapeutic siRNAs for different diseases such as cancer, obesity, diabetes, neurological disorders, and inflammatory diseases (Dunckley et al., 2005; Golkar et al., 2016; Guo et al., 2016; Kigasawa et al., 2010; Ran et al., 2014; Ryther et al., 2005; Wei et al., 2016).

Cancers of the colon and rectum (colorectal cancer) are one of the most common forms of cancer worldwide and one of the major cause of morbidity and mortality with an increased risk for patients suffering from Irritable Bowel Diseases (IBDs) such as Crohn's Disease (CD) and Colitis Ulcerosa (UC) (Burisch and Munkholm, 2015; Farraye et al., 2010; Kanaan et al., 2012; Kuipers et al., 2015; Nikolaus and Schreiber, 2007). Colorectal cancer appears to progress through a series of clinical and histopathological steps from normal to colonic epithelium crypt lesions, to benign tumors or rather adenomatous polyps, to malignant and invasive carcinomas. The identification of genetic alterations and the molecular mechanisms that underlie colorectal cancer formation is the crucial point.Whereas the key molecular factor (s) initiating cancer is unknown, different molecules, such as those involved in the pRb-E2F1 pathway, are considered to take part in the process (Ying et al., 2007). In particular, E2F1 is a transcription factor that has a key role in the promotion of the cell cycle both in normal and in cancer cells. E2F1, which level peaks in the late G1 phase of the cell cycle, promotes the transcription of numerous factors required for the progression towards the S phase of the cell cycle (Ertosun et al., 2016). Thus, E2F1 gene overexpression and/or function deregulation is related to neoplastic development in several cancers, including liver (Farra et al., 2017), gastric and colorectal carcinomas (Mega et al., 2005). In particular, in colorectal cancer, E2F1 promotes the aggressiveness of cancer cells by activating the expression of ribonucleotide reductase small subunit M2 which in turn promotes tumour growth and invasion (Fang et al., 2015).

In this study, considering the relevant role of E2F1 in colorectal cancer, a dedicate and effective delivery system for siRNAs directed against this transcription factor was developed. A siRNA already tested to be effective in silencing E2F1 -siE2F1 -(Farra et al., 2011; Dapas et al., 2009) has been encapsulated in cationic nanoliposomes purposely developed for the colon cell delivery. The novel technique used to prepare the nanoliposomes is based on lipid thin film hydration/sonication process. The technique presents several advantages over the classical methods adopted for siRNA-liposomes encapsulation and sizing processes such as fast preparation, versatility in size vesicles production, possibility to operate in sterile environments, scalability (Bochicchio et al., 2016).

The siE2F1 loaded nanoliposomes (Small Unilamellar Vesicles, SUVs) were characterized and then tested in two human colorectal cancer cell lines. Subsequently siE2F1-SUVs effectiveness was tested in intestinal human biopsy fragments, collected from healthy and IBD donors during lower endoscopy performed for colonic cancer screening.

2. Materials and methods

2.1. Unloaded and siRNA loaded vesicles preparation

2.1.1. Nanoliposomes design

To facilitate the interaction with siRNA, nanoliposomes were generated with a positive charged on the surface. To this end, the cationic dioleoyloxypropyl-*N*,*N*,*N*-trimethylammoniumpropane (DOTAP) phospholipid was embedded into the liposome structure. The charged ratio between DOTAP and siRNA sequences used was 8.5:1 (±) and it was calculated counting 1 positive charge for each DOTAP molecule and 42 negative charges for each 21 bp long siRNA molecule. This ratio was chosen on the basis of results of previous work in which the effect of different DOTAP/dsDNA (simulating "Homo sapiens siRNA probe Luciferase", 12833.4 g/mol) charge ratios on dsDNA encapsulation efficiency was studied (Bochicchio et al., 2017).

2.1.2. Nanoliposomes production

For unloaded and siRNA-loaded vesicles production, Cholesterol (CAS 57-88-5), L-α-phosphatidylcholine (PC) from egg yolk (CAS 8002-43-5) and DOTAP (CAS 132172-63-1, >99% pure) were purchased from Sigma-Aldrich (Milan, Italy). The siRNA directed against E2F1 mRNA (siE2F1, Eurofins Genomics (Ebersberg, Germany) and the control siRNA directed against the luciferase