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Research paper

Enhanced efficacy of anti-miR-191 delivery through stearylamine liposome formulation for the treatment of breast cancer cells

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ABSTRACT

MicroRNAs are gaining rapid attention as promising targets for cancer treatment; however, efficient delivery of therapeutic miRNA or anti-miRNA into cancer cells remains a major challenge. Our previous work identified miR-191 as an oncogenic miRNA overexpressed in breast cancer that assists in progression of malignant transformation. Thus, inhibition of miR-191 using antisense miR-191 (anti-miR-191) has immense therapeutic potential. Here, we have developed a stearylamine (SA) based cationic liposome for delivery of miR-191 inhibitor (anti-miR-191), and studied its efficacy in breast cancer cells (MCF-7 and ZR-75-1) in culture. SA liposomes alone inhibited cancer cell growth with lesser IC_{50s} (50% inhibitory concentration) values as compared to normal mouse fibroblast cells (L929). The efficient delivery of anti-miR-191 in SA liposome complex was found to be highly effective in killing the cancer cells than a comparable dose of SA free anti-miR-191 liposome complex. The formulation also showed negligible cytotoxicity in human erythrocytes. Combined treatment of SA liposome with anti-miR-191 markedly enhanced apoptotic cell death and suppressed the migration of cancer cells *in vitro*. Notably, anti-miR-191 loaded SA liposome complex increased chemosensitivity of breast cancer cells to currently used anti-cancer drugs (doxorubicin or cisplatin) in free form. Our work demonstrates that anti-miR-191 loaded in SA liposome complex has promising clinical application for breast cancer therapy.

1. Introduction

Breast cancer is the most frequently diagnosed and second most common recalcitrant malignancy in women around the world (Siegel et al., 2016). Despite tremendous advances, breast cancer treatment still remains a major challenge due to multiple factors such as metastasis and development of chemoresistance (O'Reilly et al., 2015). Therefore, development of more effective molecular therapeutic agents is important to enhance the clinical outcome of breast cancer treatment.

MicroRNAs (miRNAs) are small non-coding RNA molecules (19–25 nucleotides) that act as post transcriptional gene regulators by binding to the complementary sites in the 3′UTR region of the mRNA resulting in either mRNA degradation or inhibition of translation through different mechanisms (Bartel and Chen, 2004). Evidently, around 24,000 reports have shown that oncogenic miRNAs (oncomiRs) are significant key players in tumorigenesis (Ganju et al., 2017). OncomiRs are highly expressed in breast cancer and promote cancer progression by targeting cell cycle regulators and pro-apoptotic genes (Wang et al., 2015). Studies have demonstrated that overexpression of a microRNA, miR-191, in various types of cancers such as breast, colon, hepatic and gastric cancer (Peng et al., 2014; Volinia et al., 2006). Functional analyses of miR-191 identified it as an oncogenic miRNA which promotes cell proliferation, metastasis and stress resistance in breast cancer cells (Nagpal and Kulshreshtha, 2014). Thus, knockdown of endogenous miR-191 using antisense oligonucleotides (anti-miR-191) could effectively impair cancer growth suggesting anti-miR-191 (antagomirs) therapy as a promising therapeutic approach for breast cancer in clinic.

The development of miRNA-based therapeutics involves both miRNA antagonists and mimics, which attributes in loss or gain, respectively of miRNA function. Despite the strong anticancer potential of antisense miRNAs, the major challenge lies in rapid degradation and short half-life of synthetic naked antagomirs in plasma, off-target effects, repeated injection at higher dosages and poor intracellular uptake across cell bilayers (Mitchell et al., 2008;

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Pecot et al., 2011). Therefore, there is a compelling need to develop encapsulation systems to efficiently deliver antagomirs to the intracellular targets of tumors. Previous studies have shown that lipid based delivery vehicles protect and deliver nucleic acids and anticancer drugs with improved therapeutic index and reduced toxicity (Ashizawa and Cortes, 2015; Park, 2002). Notably, delivery of therapeutic agents through nano sized vehicles helps in overcoming tumor drug resistance and prevents recurrence (Liang et al., 2010; Yuan et al., 2017). Majority of anticancer agents encapsulated in liposomal formulations are in different phases of clinical trials and available commercially (Chang and Yeh, 2012; Peer et al., 2007). Importantly, surface modification of liposomes and other polymeric nanoparticles enables improved delivery of therapeutic miRNA/antisense miRNA against various cancers (Ganju et al., 2017). Recently, the first successful miRNA delivery (miR-34) through liposomes (MRX34) has completed phase I clinical trials in treating patients with melanoma, renal and hepatocellular cancer (Ganju et al., 2017; Liu et al., 2011). Cationic lipid vesicles have gained great attention in gene delivery mainly due to charge based interactions with negatively charged nucleic acid by facilitating high loading efficiency and efficient transfection reagents (Ewert et al., 2010; Zhang et al., 2015). Stearylamine (SA) bearing positively charged liposome alone has been shown to stimulate a strong leishmanicidal and trypanocidal activity mainly by damaging cell membrane of parasites (Dey et al., 2000; Yoshihara et al., 1987) and as vaccine adjuvant (Sasaki, 2014). Stearylamine based liposomes at therapeutic dosages act as strong anti-parasitic agent without affecting host cells and modulates the immune responses under pathological conditions (Banerjee et al., 2008a, 2011; Rajendran et al., 2015). Since stearylamine liposome is biodegradable and biocompatible and shows reduced systemic effects it can be used as an efficient delivery agent and chemotherapeutics. Herein, we demonstrate the use of stearylamine liposome alone as an anticancer agent and its use as an efficient agent for delivery of antisense miR-191 to breast cancer cells in culture. Based on our results, we propose that combined treatment of SA liposome anti-miR-191 complex alone or in combination with potent anticancer drugs (doxorubicin or cisplatin) in free form could be a promising approach for breast cancer therapy.

2. Material and methods

2.1. Materials

Soybean phosphatidylcholine (SPC) was purchased from Lipoid S 100, Germany. Cholesterol (Chol), stearylamine (SA), coumarin-6 (C-6), octadecyl rhodamine B (R18), DAPI (4,6-diamidino-2-phenylindole), MTT (Thiazolyl Blue Tetrazolium Bromide), doxorubicin hydrochloride, cisplatin and antisense-miR-191 (custom synthesized), fetal bovine serum (FBS), penicillin, streptomycin, other supplements were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were analytical-grade products.

2.2. Cell culture

The human breast cancer cell lines (MCF-7, ZR-75-1) and mouse fibroblast cell line (L929) were procured from Cell Repository of National Center for Cell Sciences (NCCS), Pune, India. Cell lines MCF-7, ZR-75-1 were maintained in RPMI 1640 (GIBCO) medium and for L929 cells DMEM (GIBCO) medium was used. The RPMI and DMEM medium were supplemented with 100U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum and maintained at 37 °C in a fully humidified atmosphere.

2.3. Preparation of liposomes

Liposomes were prepared using soya phosphatidylcholine (SPC) and cholesterol (Chol) in a molar ratio of 7:3 by hand shaken method. Briefly, the lipids (total 100 μmol) were dissolved in a 5 ml chloroform solution. To prepare stearylamine (SA) liposome (cationic) formulation 10 mol% was added during the preparation of lipid film. The dried lipid film so obtained was desiccated overnight and then was hydrated with 1 ml of sterile phosphate-buffered saline (PBS) containing anti-miR-191 (100 nmol), stored under a nitrogen atmosphere, and sonicated for 30 min. For fluorescent labeling of the liposomes, hydrophobic flurophores coumarin-6 (C-6) and octadecylrhodamine B (R18) was dissolved, along with lipids containing 1mol% in each formulation. The unentrapped flurophores was separated from liposomal suspension by centrifugation at 1,956 × *g* and 4 °C for 15 min and further subjected to ultracentrifugation at 11,800 × g for 1 h.

2.4. Transmission electron microscopy of SA liposomal anti-miR-191 complex

The sample preparation was performed by taking 10 μl of diluted liposomal suspension placed upon 300-mesh carbon film coated copper grid under sterile conditions and air dried prior to analysis (Polysciences, Warrington, PA). The liposomes were visualized under a Tecnai G2 T30 U-TWIN electron microscope, and images were obtained using digital imaging software at different magnifications.

2.5. Determination of liposomal size distribution and zeta potential

The average particle size and zeta potential of the liposomes were measured using a Zetasizer Nano ZS (Malvern Instruments Ltd, UK). About 10 μl of freshly prepared liposome suspension was redispersed in 990 μl of distilled water and placed in a clear disposable zeta cell (DTS-1060C). The parameters set for analyses were a scattering angle of 90 °C and a temperature of 25 °C. For each sample, the mean diameter and the standard deviation of 10 determinations were calculated using multimodal analysis. The zeta potential was measured by a combination of laser Doppler velocimetry and phase analysis light scattering (M3-PALS) technique at 25 °C.

2.6. Serum stability and the in vitro release of anti-miR-191 loaded SA liposome complex

Briefly, samples of naked anti-miR-191 in aqueous solution or the SA Lip anti-miR-191 complex were mixed in a 1:1 ratio with fresh FBS (50% serum) and incubated at 37 °C for the indicated time points. At different incubation time, aliquots (20 μl) from each mixture of the naked antimiR-191 or the SA Lip complex were mixed with (5 μl) 2% SDS and (2.5 μl) 10% glycerine and incubated for 30 min. The mixture was loaded onto a 1% agarose gel containing ethidium bromide. Further, to determine the *in vitro* release profile of anti-miR-191 from SA liposomal complex was performed at either acidic pH 6.4 or neutral pH 7.4 at 37 °C under moderate shaking. At each time points samples were taken and were substituted with same volume of fresh corresponding pH buffer. The concentration of released fraction of anti-miR-191 was determined by a semiquantitative gel electrophoresis method at each condition using ImageJ software (Cao et al., 2013). The loading efficiency of anti-miR-191 in SA liposome complex was determined as reported by (Silva et al., 2012, 2016).