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Polymeric micelles containing reversibly phospholipid-modified anti-survivin siRNA: A promising strategy to overcome drug resistance in cancer

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ABSTRACT

The discovery that survivin, a small anti-apoptotic protein, is involved in chemoresistance, opens a new scenario to overcome the drug resistance in cancer. It was shown that siRNA can efficiently inhibit the expression of survivin in cancer cells. However, the clinical use of siRNA is still hampered by an unfavorable pharmacokinetic profile. To address this problem, earlier we developed a novel system to deliver siRNA into cancer cells. Namely, we reversibly modified the survivin siRNA with a phosphothioethanol (PE) portion via a reducible disulfide bond and incorporated the resulting siRNA-S-S-PE conjugate into nanosized polyethyelene glycol 2000-phosphatidyl ethanolamine (PEG_{2000}-PE)-based polymeric micelles (PM), obtaining survivin siRNA PM. The activity of these nanopreparations was evaluated by survivin protein down-regulation, tumor cell growth inhibition, and chemosensitization of the treated tumor cells to paclitaxel (PXL). We found a significant decrease of cell viability and down-regulation of survivin protein levels after treatment with survivin siRNA PM in several cancer cell lines. In addition, the down-regulation of survivin by treating cells with survivin siRNA PM, elicited a significant sensitization of the cells to PXL, in both sensitive and resistant cancer cell lines. Finally, we demonstrated successful co-delivery of PXL and survivin siRNA in the same PM leading to superior therapeutic activity compared to their sequential administration. Our results support the use of this new platform for the treatment of the most aggressive tumors.

1. Introduction

Survivin, the smallest member of the inhibitors of apoptosis (IAP) family, has gained much attention in recent years as a promising new target in cancer therapy due to its differential expression in tumours compared to normal tissues [1]. Survivin plays an important role in the negative regulation of apoptosis as well as in cell division [2,3]. Moreover, survivin expression in malignant tissues has been correlated with drug resistance [4]. Accordingly, inhibition of survivin has been of clear interest for cancer therapy. In the last years, many researchers have proposed various ways to counteract survivin activity in cancer cells with the aim to inhibit the tumor growth potential and to sensitize the tumor cells to chemotherapeutic agents. RNA interference (RNAi) offers an attractive and powerful approach to efficiently inhibit survivin expression in cancer cells [5]. A. Carvalho et al. [6] were the first to use siRNA to suppress survivin levels in HeLa cells, showing a specific depletion of survivin for at least 60 h after the transfection with a specific siRNA. Seth et al. have demonstrated the *in vivo* silencing of survivin and a significant dose-dependent decrease of tumor volumes after intravesical instillation of liposomes containing survivin siRNA in an animal model of bladder cancer [7]. Despite all the potential of siRNA in cancer treatment, selective inhibition of an overexpressed gene via RNAi requires an effective delivery strategy that ameliorates the significant issues associated with its pharmacokinetic profile. In particular, the poor stability in biological fluids and the low cellular uptake impaired siRNA direct use in clinical trials. In the literature, several approaches for siRNA delivery *in vitro* and *in vivo*, such as viral vectors [8,9], hydrodynamic injection [10], cationic polymer and lipids [11,12] are reported. However, only few have demonstrated clinical applicability due to toxicity and poor stability in biological fluids. Therefore, the transition of siRNA-based approach to the clinical setting requires the development of a suitable delivery system.

We previously reported bio-reductive PM for siRNA delivery based on siRNA conjugated to PE via a disulfide linkage [13]. This strategy is based on the dramatically higher concentration of reductases in the tumor microenvironment over normal tissues [14] as well as glutathione inside cancer cells [15]. Chemical

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conjugation of siRNA and incorporation of siRNA conjugate into PM offers the advantage of protecting the siRNA from degradation *in vitro* by a facile reaction and at the same time, the cleavable disulfide bonds linked to the siRNA, allow to liberate it free when inside the cell for target-specific gene silencing. Thus, the conjugated siRNA can be incorporated via the PE moiety into a non toxic delivery system, such as PEG_{2000}-PE-based PM [16], to become stable in physiological conditions and able accumulate in the areas with an abnormal vascularization, i.e. tumors, via the enhanced permeability and retention (EPR) effect.

Here, we formulated nanosized PEG_{2000}-PE PM for anti-survivin siRNA delivery. *In vitro* cytotoxicity and survivin protein levels studies revealed the ability of survivin siRNA PM to inhibit efficiently the cellular growth and to down-regulate the survivin in different cancer cell lines. In a second phase, we investigated the potential of combination therapy with survivin siRNA and a chemotherapeutic agent, PXL. PXL exhibits its anticancer activity by promoting tubulin polymerization and stabilizing microtubules, which results in mitotic G2/M arrest and apoptosis [17]. The clinical effectiveness of PXL, an agent widely used in clinic for the treatment of several tumors, is often hampered by acquired drug resistance [18]. Since sensitization to PXL by survivin down-regulation has been reported [19–21] we evaluated co-treatments with PXL and anti survivin siRNA. Cells were either subjected to survivin siRNA before PXL treatment or treated with PM simultaneously encapsulating PXL and survivin siRNA. Our results suggest that the developed survivin siRNA PM greatly sensitize the cells to PXL treatment and the simultaneous delivery of survivin siRNA and PXL, by using PM, significantly enhances the tumor response to PXL in resistant cancer cell lines.

2. Materials and methods

Survivin siRNA with the following sense sequence 5′-GCAUUCGUCCGGUUGCGCUdTdT-3′ and a scrambled siRNA with the following sense sequence 5′-AUGAACUUCAGGGUCAGCUdTdT-3′ have been used. Both siRNAs modified at the 3′-end of the sense strand with N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) group were purchased from Thermo Scientific Dharmacon (Pittsburgh PA, USA). The paclitaxel (PXL) was purchased from LC Laboratories (Woburn MA, United States). The 1,2-dipalmitoyl-sn-glycero-3-phosphothioethanol (PE-SH, MW 731) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(poly(ethylene glycol))-2000] (PEG_{2000}-PE) were from Avanti Polar Lipids (Alabaster, AL). The d-Salt dextran desalting column was from Pierce (Rockford, IL, USA). Acetonitrile (HPLC grade), analytical grade chloroform (CH3Cl), DMSO, methanol (MeOH) and Triton X-100 were supplied by Sigma Aldrich (Saint Louis, MO). The human total survivin immunoassay, Surveyor IC, was purchased from R&D System (Minneapolis, MN). RNase/DNase-free water was obtained from MP Biomedicals (Solon, OH), the phosphate saline buffer (PBS) 10 × solution and bovine serum albumin (Fraction V) were from Fisher Scientific (Fair Lawn, NJ). β-tubulin antibody (G-8) was from Santa Cruz Biotechnology (Dallas, Texas, USA). Fluorescein (FITC)-conjugated AffiniPure Donkey Anti-Mouse IgG (H + L) was provided by Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Hoechst 33342 trihydrochloride, trihydrate, was purchased from Molecular Probes (Eugene, Oregon, USA). Vecta Shield mounting medium for fluorescence, H-1000, was from Vector Laboratories, Inc. (Burlingame, CA).

2.1. Cell culture

Human ovarian cancer cell line (A2780) and human breast cancer cell line (MDA-MB231) were cultured in DMEM medium, containing 10% fetal bovine serum (FBS), 100 U/mL penicillin G sodium and 100 mg/mL streptomycin sulfate (complete medium), in a humidified atmosphere of 95% air 5% CO_2 at 37 °C. Human ovarian cancer cell line sensitive (SKOV3) and multi drug resistant (MDR) (SKOV3-tr) were grown in the complete RPMI1640 medium. The SKOV3-tr cells have been widely characterized and are known to overexpress the MDR-1 gene (Duan et al., 1999). All the cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Trypan Blue solution and trypsin were from CellGro (Kansas City, MO).

2.2. Synthesis of survivin siRNA-S−S-PE conjugate

The survivin siRNA-S−S-PE conjugate was synthesized as previously described by Musacchio et al. [13]. Briefly, an aqueous solution of the SPDP-activated siRNA (20 nmol in 120 μL of RNase/DNase-free water), was added dropwise to a solution of PE-SH (2 μmol) in DMSO and $CHCl_3$ (total volume of organic solvents 350 μL). The reaction was carried out for 48 h at room temperature with continuous stirring. The un-reacted reagents were removed by desalting column. The collected samples containing the survivin siRNA-S-S-PE conjugate were freeze-dried for overnight. After freeze-drying, the survivin siRNA-S-S-PE conjugate was hydrated with PBS pH 7.4 at a final siRNA concentration of 20 nmol/mL and ultracentrifuged for 1 min at 14.5 × 1000 rpm to further remove mixed solvents and/or PE-SH. The survivin siRNA-S–S-PE conjugate was stored at −20 °C. The conjugation efficiency and the amount of survivin siRNA-S−S-PE conjugate was determined, after purification, by absorbance at 260 nm using a Nanodrop (2000c Spectrophotometers, Thermo Scientific). Scrambled siRNA was modified following the same protocol.

2.3. Incorporation of siRNA-S−S-PE in PEG_{2000}-PE Micelles

The PEG_{2000}-PE micelles containing siRNA-S-S-PE were prepared by hydration of a thin polymeric film [16]. In particular, PEG_{2000}-PE was dissolved in chloroform (20 mg/mL) and the resulting solution was added to a 50 mL round-bottom flask. The organic solvent was removed under reduced pressure by a rotary evaporator under nitrogen atmosphere, followed by freeze-drying. Then, the polymeric film was hydrated with 1 mL of survivin siRNA-S-S-PE in phosphate buffer at pH 7.4 at different PEG_{2000}-PE/siRNA-S-S-PE weight ratio (1:200, 1:500, 1:750). The resulting dispersion was gently vortexed to form mixed micelles, so-called survivin siRNA PM. PEG_{2000}-PE-based PM containing scrambled siRNA-S-S-PE and plain PM were prepared similarly. Each formulation was prepared in triplicate.

2.4. Co-encapsulation of PXL and survivin siRNA-S−S-PE in PEG_{2000}-PE PM

PXL was incorporated in survivin siRNA PM as follow. Briefly, an organic solution of PXL in methanol (1 mg/mL) was added to the PEG_{2000}-PE mixture in chloroform. The initial loading of PXL into micelles was 1% w/w. The incorporation of survivin siRNA-S–S-PE into these micelles was determined as reported above. The encapsulation efficiency of PXL in PM was determined as reported by Musacchio et al. [22].

2.5. Characterization of PM

The mean diameter of PM containing survivin siRNA-S-S-PE alone or in combination with PXL, was determined at 20 °C by the dynamic light scattering (DLS) using a Zeta Plus Instrument (Brookhaven Instrument Co., Holtsville, NY). Briefly, each sample was diluted in deionizer/filtered water and analyzed with detector at 90° angle. As a measure of the particle size distribution, polydispersity index (P.I.) was used. For each batch, mean diameter and size distribution were the mean of three measures. For each formulation, the mean diameter and P.I. were calculated as the mean of three different batches.

2.6. siRNA-S-S-PE conjugate incorporation in PEG_{2000}-PE PM

The quantitative analysis of siRNA-S-S-PE in PM was performed by the size-exclusion high-performance liquid chromatography (SEC-HPLC). For the analysis, the HPLC system (D-7000 HPLC, Hitachi, Japan) equipped with a Shodex protein KW-804 column (Showa Denko, Japan) and an UV detection at 280 nm, was used. The mobile phase was composed of 50 mM NaCl and 50 mM Tris–HCl (pH 8.0) and the flow rate at 1.0 mL/min. The siRNA-S-S-PE loading efficiency into PM was evaluated by ratio of the area under the peaks at the same retention time (tr ca. 10 min) of survivin siRNA-S-S-PE not encapsulated in PM and the survivin siRNA-S-S-PE initially added to the PM. As a control, plain PM were also analyzed. To confirm the data collected by SEC-HPLC, we analyzed the same samples also by the reverse phase HPLC (RP-HPLC). The RP-HPLC analysis was carried out as reported by Musacchio et al. [13]. Then, we evaluated the loading efficiency of PXL in survivin siRNA PM. The quantitative analysis of PXL was determined by RP-HPLC as reported by Musacchio et al. [22], using a XBridge column (4.6 mm × 250 mm, Waters, Milliford, USA). The mobile phase consisted of water and acetonitrile with volume ratio 60:40, the elution was performed at a rate of 1.0 mL/min, and PXL was detected from injected sample (50 μL) at 227 nm.

2.7. Cell viability assay

A2780, MDA-MB 231, SKOV3 and SKOV3-tr cells were seeded at a density of 3×10^3 cells/well in 96-well culture plates for 24 h. After 24 h, the cells were treated with various concentrations of survivin siRNA-S–S-PE free or in PM, scrambled siRNA-S-S-PE in PM and plain PM, in serum-contained media. The final concentration of siRNA-S-S-PE was in the range of 200–17.6 nM. After 6 h, the medium was replaced with fresh medium, and the cells were incubated until the 48-h-time point was reached. Cells without treatment were used as control. The cell viability was determined by Cell Titer Blue assay following manufacturer's protocol. The experiments were done in triplicate on three different sample preparations.