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Efficacy of gemcitabine conjugated and miRNA-205 complexed micelles for treatment of advanced pancreatic cancer

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ABSTRACT

Clinical effectiveness of gemcitabine in pancreatic cancer is hindered due to its rapid plasma metabolism and development of chemo-resistance. We have previously delineated the significant role of miRNAs in mediating the growth and proliferation of cancer stem cells (CSCs) which in turn result in chemo-resistance, invasion and metastasis. Here, we designed self-assembling, gemcitabine conjugated cationic copolymers for co-delivery of a tumor suppressor miRNA-205 (miR-205) and evaluated their *in vivo* efficacy in a pancreatic cancer ectopic tumor model developed using gemcitabine resistant MIA PaCa-2^R cells. Combination formulations showed mean a particle size of <100 nm and gemcitabine payload of >10% w/w, exhibited miRNA complexation at N/P ratio of 4/1, sustained release of gemcitabine for >10 days, transfection efficiency of >90%, extended miRNA and drug stability in serum. Functional assays in gemcitabine resistant MIA PaCa-2^R and CAPAN-1^R pancreatic cancer cells revealed that the combination formulations effectively reversed chemo-resistance, invasion and migration. In pancreatic tumor model, the combination formulation treated group showed significant inhibition of tumor growth. Immuno-hiostochemical analysis revealed decreased tumor cell proliferation with increased apoptosis in the animals treated with miR-205 and gemcitabine combination.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal malignancies being the fourth leading cause of cancer-related deaths in the United States, with a 5 year survival rate of <5% and a median overall survival of <6 months [1,2]. It has a very grim prognosis since the majority of patients already have either locally advanced or metastatic cancer, when they are diagnosed. Only 10–20% of PDACs are suitable for surgical resection at initial diagnosis, while the therapeutic options in a majority of patients eventually become restricted to an intensive regimen mainly with gemcitabine- or fluorouracil-based systemic chemotherapy [3,4]. Gemcitabine received regulatory approval for treatment of pancreatic cancer, based on a randomized trial in the mid-1990s [5]. It displays its anticancer activity by inducing S-phase arrest and inhibiting DNA synthesis [6,7]. However, its clinical application is severely limited by its rapid metabolism to inactive metabolite, 2, 2′-difluorodeoxyuridine (dFdU), resulting in a short plasma half-life of 8–17 min [8]. In addition, its hydrophilic nature creates formulation challenge and its penetration into the pancreatic cancer tissue which has a very dense stroma [9].

Clinical data shows that therapeutic benefits were experienced by only 23.8% of gemcitabine-treated patients in their early stages of treatment. Thereafter, PDAC developed resistance to gemcitabine, resulting in a mere 6 months of median overall survival [3–5]. To improve the overall survival, several multidrug combinations with gemcitabine are being investigated in pancreatic cancer clinical trials. Results from phase II studies of gemcitabine combined with cisplatin, 5-fluorouracil (5-FU), irinotecan, and oxaliplatin, in advanced and metastatic pancreatic cancer have suggested that efficacy could be improved [10,11]. However, similar benefit was not evident in phase III trials [12,13]. Chemotherapy with nanoparticle albumin bound (nab)-paclitaxel and gemcitabine in patients with metastatic pancreatic cancer has shown a median overall survival of 8.5 months compared to 6.7 months for patients treated with gemcitabine alone and post-treatment one-year survival rate of 35% of patients who received both drugs while 22% of those who received gemcitabine alone [14]. Although combinations

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like FOLFIRINOX are showing significant improvement over gemcitabine monotherapy; the benefit is still limited to a moderate improvement which might be attributed to the emergence of resistance due to prolonged treatment and also due to the dense impermeable tumor microenvironment resulting from the proliferation of fibroblasts and increased stromal fibrosis [15,16].

In our previous study, we prepared gemcitabine-conjugated polymeric micelles which successfully delivered a therapeutic pay-load of gemcitabine in an ectopic model of pancreatic cancer [17]. Although this treatment exhibited higher efficacy compared to aqueous gemcitabine solution, this micellar conjugate of gemcitabine was fraught with the emergence of chemo-resistance. Understanding that how PDAC acquires resistance to gemcitabine is important for the development of therapies which could enhance its efficacy. We and others have identified the role of CSCs which are regulated by 17–25 nucleotide non-coding RNAs known as miRNA in mediating chemo-resistance, metastasis and invasion [18–21]. CSCs are a small percentage of cells (1–2%) in the tumor mass characterized by unique features such as unlimited self-renewal, long lifespan, high metastatic potential, and resistance to chemotherapy [18,20]. Targeting CSCs is a promising strategy however, the efficacy of currently available CSC-targeting therapies is far from satisfactory. We have previously shown that miRNAs can modulate the tumor characteristics and in fact these could be used as therapeutic intervention [18]. Our studies in resistant pancreatic cancer cells and primary pancreatic cancer tumor specimens confirmed the aberrant expression of miR-205 in PDAC and indicated its potential to chemosensitize gemcitabine resistant cancer cells [18]. However, *in vivo* delivery of miRNAs remains a challenge due to several barriers such as their poor biostability, rapid excretion, poor cellular uptake and improper intracellular release [22]. Cationic carriers such as conjugates [23,24], inorganic nanoparticles [25,26], hydrogels [27,28] and cationic liposomes [29,30] have been developed for nucleic acid delivery which can form complexes via electrostatic interactions and thus render protection in the hostile biological environment. However, such systems are limited to carrying only nucleic acids. A carrier system that can simultaneously carry miRNA and a small molecule drug could be advantageous. For this purpose, biodegradable polymeric carriers could be of enormous interest since they can be tailored to carry the miRNA and anticancer small molecule to the target site.

In the present study, we have developed polymeric formulation for co-delivery of miR-205 mimic and gemcitabine to target both bulk tumor cells and resistant CSCs. For this, gemcitabine conjugated cationic copolymers, poly(ethyleneglycol)-block-poly(2-methyl-2-carboxyl-propylenecarbonate-graft-dodecanol-graft-cationic ligands) (mPEG-b-PCC-g-GEM-g-DC-g-CAT), were synthesized and used for complex formation with a synthetic miR-205 mimic. Formulations containing gemcitabine and miR-205 mimic with different cationic pendent groups such as N,N-dimethyldiisopropylenetriamine (DP), spermine (SP) and tetraethylenepentamine (TP) were prepared and characterized for particle size, zeta potential, gemcitabine pay-load, miRNA complexation, stability and transfection efficiency. The formulations were subsequently tested for reversal of chemo-resistance, migration and invasion in gemcitabine resistant pancreatic cancer cell lines (MIA PaCa-2^R and CAPAN-1^R). The optimized combination formulation was then tested for *in vivo* efficacy in ectopic tumor model of pancreatic cancer after intratumoral injection and evaluated based on reduction in tumor growth, changes in body weight and immunohistochemistry of tumor samples.

2. Materials and methods

Gemcitabine hydrochloride was purchased from LC Laboratories (Woburn, MA). mirVana™ miRNA mimic (hsa-miR-205-5p), mirVana™ miRNA mimic negative control #1 and BLOCK-iT™ Fluorescent Oligo were purchased from Life Technologies (Carlsbad, CA). 2,2-Bis(hydroxymethyl) propionic acid, methoxy polyethyleneglycol (mPEG; Mn = 5000 Da, polydispersity index (PDI) = 1.03), stannous 2-ethylhexanoate ($Sn(Oct)_2$), tetraethylenepentamine, spermine, N,N-dimethyldipropylenetriamine, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide HCl (EDC), 1-hydroxybenzotriazole (HOBT) and benzyl bromide were purchased from Aldrich (St. Louis, MO) and used as received. Primers for gene expression analysis were purchased from Integrated DNA Technologies (Coralville, IA). All other chemicals were of analytical grade and used as it is without further purification.

2.1. Synthesis of monomer and copolymers

Monomer 2-methyl-2-benzyloxycarbonyl-propylene carbonate (MBC) and its copolymer with mPEG (mPEG-MBC) were synthesized by ring opening polymerization followed by reduction reaction to obtain the copolymer containing carboxyl pendent groups (mPEG-PCC) [17]. Gemcitabine and dodecanol were conjugated to the copolymer mPEG-PCC by carbodiimide coupling as we have reported earlier [17]. To make this conjugate amenable to complex formation with miRNA, cationic chains (tetraethylenepentamine; TP, spermine; SP or dimethyldipropylenetriamine; DP) were further introduced into the polymer backbone mixture and allowed to stir for 24 h at room temperature under nitrogen atmosphere. Gemcitabine conjugated cationic polymer (mPEG-b-PCC-g-GEM-g-DC-g-CAT, where GEM is gemcitabine, DC is dodecanol and CAT is cationic chain) so obtained was purified by dissolving in chloroform and repeated precipitation in isopropyl alcohol followed by diethyl ether. The purified polymer thus obtained was dried under vacuum, dissolved in acetone and dialyzed extensively against distilled water followed by lyophilization to obtain the dried product. Polymers were characterized using ^{1}H NMR (Bruker 400 MHz, $T = 25$ °C; DMSO-d6 solvent), FTIR and nitrogen content analyzer.

2.2. Formulation and characterization

Gemcitabine conjugated, miR-205 complexed, micelles were prepared by film hydration method as reported earlier with slight modification [31]. Briefly, 10 mg of gemcitabine conjugated cationic copolymer (mPEG-b-PCC-g-GEM-g-DC-g-CAT) was dissolved in chloroform (0.2 ml) followed by evaporation under reduced pressure to form a thin film. HEPES buffer (10 mM, pH 6.5, 1 ml) containing miR-205 was then added to hydrate the film and solution was vortexed vigorously for 1 min and allowed to stand for 30 min at 37 °C to enable miRNA complexation. It was then centrifuged at 5000 g for 5 min, filtered through 0.22 μm filter (Millipore) and lyophilized to obtain the final formulation.

Mean particle size, size distribution and zeta potential of polyplexes (1 mg/ml in 10 mM HEPES buffer, pH 6.5) were measured using Malvern Zetasizer (NanoZS Series). Gemcitabine content in the polyplexes was measured by HPLC-UV analytical method after extraction of conjugated drug by alkaline hydrolysis as reported earlier [17]. Agarose gel retardation assay was carried out to determine the appropriate N/P ratio resulting in effective complex formation between miR-205 and the copolymer. The polyplexes were incubated for 30 min at 37 °C, run on 1% w/v agarose gels containing 0.05 mg/ml ethidium bromide at 100 V for 30 min. Gels were photographed under UV illumination using GelDoc Ez system (Bio-Rad, Hercules, CA). To determine the ability of polyplex to release the complexed miRNA (miRNA dissociation), polyanion competition assay was carried out using heparin sulfate (170 U/mg). Polyplexes were prepared at N/P ratio of 16/1 to ensure complete binding followed by incubation with heparin sulfate solution for 45 min at 37 °C using different weight ratios of heparin to miRNA. The solutions were then run on agarose gels and visualized by UV illumination using GelDoc Ez system (Bio-Rad, Hercules, CA).

2.3. In vitro gemcitabine release from the combination formulation

To determine gemcitabine release from the micelles, formulations (~0.5 mg gemcitabine) were prepared and transferred to a dialysis bag (molecular weight cutoff 2000 Da), clamped at both ends, and placed in aqueous phosphate buffered saline (PBS; pH-5.5 and 7.4; 5 ml). Samples (0.5 ml) were withdrawn at regular time intervals and replaced with an equivalent amount of the fresh media. Gemcitabine content in the samples was measured and cumulative gemcitabine release was plotted against time. To determine the effect of miRNA co-presence on gemcitabine release, studies were performed in the presence and absence of miRNA in the formulation.

2.4. miRNA stability in serum

We have previously reported that gemcitabine after conjugation to the copolymer exhibits serum stability [17]. To determine the stability of miRNA against serum degradation, polyplexes with gemcitabine conjugated cationic copolymers were prepared and incubated with 25% fetal bovine serum for 24 h at 37 °C followed by dissociation using heparin sulfate for 45 min to ensure complete release of miRNA from the formulations and run on agarose gel. miRNA bands were digitized and quantified using ImageJ analysis software to determine the mean density of the miRNA bands. All data is reported as mean of three independent experiments.

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