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Polyplex-mediated inhibition of chemokine receptor CXCR4 and chromatin-remodeling enzyme NCOA3 impedes pancreatic cancer progression and metastasis

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Abstract

Pancreatic cancer (PC) is one of the most aggressive malignancies due to intense desmoplasia, extreme hypoxia and inherent chemoresistance. Studies have implicated the expression of chemokine receptor CXCR4 and nuclear receptor co-activator-3 (NCOA3) in the development of desmoplasia and metastatic spread of PC. Using a series of polymeric CXCR4 antagonists (PCX), we optimized formulation of PCX/siNCOA3 polyplexes to simultaneously target CXCR4 and NCOA3 in PC. Cholesterol-modified PCX showed maximum CXCR4 antagonism, NCOA3 silencing and inhibition of PC cell migration *in vitro*. The optimized PCX/siNCOA3 polyplexes were used in evaluating antitumor and antimetastatic activity in orthotopic mouse model of metastatic PC. The polyplexes displayed significant inhibition of primary tumor growth, which was accompanied by a decrease in tumor necrosis and increased tumor perfusion. The polyplexes also showed significant antimetastatic effect and effective suppression of metastasis to distant organs. Overall, dual-function PCX/siNCOA3 polyplexes can effectively regulate tumor microenvironment to decrease progression and dissemination of PC.

Keywords

Polyplexes; siRNA delivery; Pancreatic cancer; Metastasis; CXCR4; NCOA3; Mucin; Tumor perfusion

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1. INTRODUCTION

Pancreatic cancer (PC) is predicted to become the second leading cause of cancer-related mortalities by 2030 [1]. This grim prognosis underscores the urgent need to develop better therapeutic strategies. The poor outlook for PC is due to late diagnosis, early metastasis, and complex tumor microenvironment that contribute to inherent resistance to available therapies and severely limit drug delivery [2-4]. Aberrant mucin expression is one of the characteristic features of PC that has been directly implicated in the PC pathobiology [2, 5-6]. Mucins interact with receptor tyrosine kinase and extracellular matrix proteins, and signal through their cytoplasmic tail to facilitate PC progression, metastasis, and chemoresistance [7-8].

We have previously identified nuclear receptor coactivator-3 (NCOA3), a chromatin remodeling enzyme, as a critical modulator of the expression of mucins in PC [7]. Silencing of NCOA3 in PC cells downregulates the expression of two mucins, MUC1 and MUC4, that are critical for PC progression. As a member of the p160SRC family of proteins, NCOA3 interacts with nuclear receptors and transcription factors [9]. It remodels chromatin for active transcription due to its intrinsic histone-acetyltransferase activity [9]. NCOA3 is undetectable in normal pancreas but its expression appears early during PC initiation, corresponding with mucin expression and is also elevated in lung, lymph and liver metastatic lesions [7]. In addition to mucins, NCOA3 upregulates the expression of multiple chemokines that are responsible for the recruitment of immune cells to pancreatic tumors, perpetuation of pro-inflammatory conditions, and activation of pancreatic stellate cells (Scheme 1). Among various chemokine pathways associated with the pathology of PC, the CXCR4/SDF1 axis plays critical role in the invasion and metastasis of PC [10-12]. The stromal cells produce abundant SDF-1, which activates CXCR4 in PC cells and results in enhanced chemotaxis, transendothelial migration, and invasion [13]. CXCR4 upregulation can also augment the Sonic Hedgehog pathway and promote stem-cell-like phenotype, enhanced desmoplasia, chemoresistance, and invasiveness of PC [14]. Multiple retrospective clinical studies have directly linked the expression of CXCR4 with poor survival and metastasis in PC patients [15, 16]. Pro-inflammatory conditions found in PC lead to activation of pancreatic stellate cells, which then secrete collagen and other extracellular matrix proteins in the tumor microenvironment. These extracellular matrix proteins are crosslinked by lysyl oxidases (LOX), which results in hardening of desmoplasia and subsequent collapse of the blood vessels, poor tumor perfusion, increased interstitial pressure, extreme hypoxia, and poor delivery of therapeutics. Importantly for this study, NCOA3 significantly upregulates the expression of LOXL2, a LOX family member associated with the aggressiveness of PC.

Multiple strategies have been explored to minimize desmoplasia as a way of improving the delivery of therapeutics to PC. Hyaluronan/CD44 and Sonic Hedgehog have been among the successfully explored key regulatory pathways that contribute to the desmoplasia in PC [17-18]. Stromal hyaluronan was ablated in autochthonous mouse model of PC using systemic administration of PEGylated recombinant hyaluronidase [19-20]. Inhibition of the Hedgehog signaling transiently increased the microvascular density and enhanced intratumoral concentration of gemcitabine, leading to transient stabilization of the disease [21]. Therapeutics based on nucleic acids, such as small interfering RNA (siRNA) and

microRNA have also been explored in the treatment of various cancers, including PC [22–24]. For example, biodegradable polyester nanoparticles were used to deliver siRNA to silence mutant K-ras, which induced apoptosis and significantly reduced the growth, migration and invasion of PC cells [25]. Hybrid lipid-polymer nanoparticles were developed to deliver siRNA against hypoxia-inducible factor 1 α (HIF1 α) with the goal of overcoming hypoxia-induced resistance to gemcitabine therapy in PC. The nanoparticles effectively co-delivered the anti-HIF1 siRNA and gemcitabine and achieved synergistic antitumor and antimetastatic effects in orthotopic PC model [26]. Successful siRNA delivery in spontaneous PC model was reported using micelles based on calcium phosphate and amphiphilic block copolymers [27]. Polymer micelles were also used to co-encapsulate a Hedgehog inhibitor and tumor-suppressor microRNA, let7b. Treatment with the combination micelles resulted in decreased tumor proliferation and enhanced apoptosis in ectopic mouse model of PC [28].

Given the critical role of NCOA3 and CXCR4 and the therapeutic challenges of PC, we designed novel neoadjuvant treatment that relies on delivery of NCOA3-silencing siRNA using polyplexes based on polymeric CXCR4 inhibitors (PCX) (Scheme 1). We hypothesized that the ability of the developed polyplexes to simultaneously downregulate mucin expression *via* NCOA3 silencing and inhibition of CXCR4 will lead to improved antitumor and antimetastatic activity.

2. MATERIALS AND METHODS

2.1. Materials

N,N'-Hexamethylenebisacrylamide (HMBA) was from Polysciences (Warrington, PA). mPEG-Acrylamide (2 kDa) was purchased from Creative PEGworks (Winston-Salem, NC). *N,N*-diisopropylethylamine (DIPEA) and trifluoroacetic acid (TFA) were from Acros Organics (Fair Lawn, NJ). Cholesteryl chloroformate and branched poly(ethyleneimine) (PEI, 25 kDa) were from Sigma-Aldrich (St. Louis, MO). AMD3100 was from Biochempartner (Shanghai, China). Dulbecco's Modified Eagle Medium (DMEM), Dulbecco's Phosphate Buffered Saline (PBS), and Fetal Bovine Serum (FBS) were from Thermo Scientific (Waltham, MA). Rotor-Gene SYBR Green RT-PCR Kit was from QIAGEN (Hilden, Germany). Non-targeting siRNA control (siScr, 5'-UUCUCCGAACGUGUCACGUUU-3') and stability enhanced NCOA3 siRNA (siNCOA3, 5'-GACAGGCACUUGAAUUGAAUU-3') were custom synthesized by GE Healthcare Dharmacon (Lafayette, CO). TRIzol[®] reagent and human CXCR4 primers (F 5'-GCATGACGGACAAGTACAGGCT-3', R 5'-AAAGTACCAGTTTGCCACGGC-3') were purchased from Life Technologies (Carlsbad, CA). Allophycocyanin (APC) mouse anti-human CXCR4 antibody and APC mouse IgG2a, κ isotype controls were from BD Biosciences (San Jose, CA). Anti-CXCR4 antibody [UMB2] was purchased from Abcam (ab124824, Cambridge, MA). Anti-NCOA3 antibody was purchased from Santa Cruz Biotechnologies (sc25742, Dallas, TX). The MUC4 monoclonal antibody (8G7) used in this study was developed by Dr. Batra group [29]. All other reagents were from Fisher Scientific and used as received unless otherwise noted.

2.2. Synthesis of polymeric CXCR4 inhibitors (PCX)

PCX used in this study were synthesized by Michael-type polyaddition of equal molar ratio of HMBA and a corresponding cyclam monomer as described in detail previously [30–32]. Briefly, to synthesize PCX-1, AMD3100 and HMBA were polymerized in methanol/water (7/3 v/v) at 37 °C for 3 days. PCX-1 was obtained by dialysis against deionized water with pH adjusted to 4 using 1M HCl (membrane molecular weight cut-off 3.5 kDa). PCX-2 was prepared by polymerization of HMBA and a Boc-protected cyclam monomer in methanol/water (7/3 v/v) for 14 days at 50 °C [33]. The Boc groups in the polymer were removed by trifluoroacetic acid and the product was dialyzed and freeze-dried. Cholesterol-modified PCX-1 (Chol-PCX) were prepared by reaction of PCX-1 with cholesteryl chloroformate in methylene chloride [30]. PEGylated PCX-1 was prepared by reaction of PCX-1 with mPEG(2kDa)-acrylamide [31]. PEG-PCX was isolated by precipitation in diethyl ether, centrifugation, and dialysis against deionized water.

2.3. Polymer characterization

Molecular weights of PCX were analyzed by size exclusion chromatography on TSKgel G3000PW_{XL}-CP column (Tosoh Bioscience) and detected using a miniDAWN TREOS multi-angle light scattering detector and a Optilab T-rEX refractive index detector from Wyatt Technology (Santa Barbara, CA). The contents of cholesterol and PEG in the copolymers were determined using ¹H-NMR.

2.4. Preparation and characterization of PCX/siRNA polyplexes

Polyplexes were prepared by mixing equal volume of siRNA and PCX solutions in HBG and then incubated at room temperature for 20 min. Hydrodynamic diameter and zeta potential were determined by dynamic light scattering using a ZEN3600 Zetasizer Nano-ZS (Malvern Instruments). Complexation of siRNA by PCX was examined by agarose gel electrophoresis. Polyplexes were prepared at different w/w ratios in HBG and loaded onto a 2% agarose gel containing 0.5 µg/mL ethidium bromide. Gels were run at 75 V in 0.5x Tris/Borate/EDTA buffer for 30 min and then imaged.

2.5. Cell culture

Human PC cell line CD18/HPAF was originally derived from the parental heterogeneous HPAF pancreatic tumor cell line by a limiting dilution technique [34]. Luciferase-expressing CD18/HPAF (CD18/HPAF.luc) cell line was obtained by transfecting CD18/HPAF cells with pbabe.puro-FlucI vector as described previously [35]. The cell line was cultured in DMEM with 1% Pen-Strep and 10% FBS. Human hepatocellular carcinoma, HepG2 cells were purchased from ATCC (Manassas, VA) and cultured in MEM supplemented with 10% FBS. Human epithelial osteosarcoma U2OS cells stably expressing functional EGFP-CXCR4 fusion protein were purchased from Fisher Scientific and cultured in DMEM supplemented with 2 mM L-glutamine, 1% Pen-Strep, 0.5 mg/ml G418 and 10% FBS. All cells were maintained in an incubator at 37 °C and 5% CO₂.

2.6. CXCR4 antagonism

Phenotypic CXCR4 redistribution assay using a high-content fluorescence microscopy analysis was used to measure CXCR4 antagonism of polyplexes using protocol described in detail previously [33]. Briefly, U2OS cells stably expressing functional EGFP-CXCR4 fusion protein were treated with polyplexes (1 µg/mL polymer, polymer/siRNA w/w ratio of 2) or AMD3100 (300 nM) for 30 min and 10 nM SDF-1α was added for 1 h. The cells were fixed and nuclei stained with Hoechst-33258. The internalization of the EGFP-CXCR4 receptors was quantified by Cellomics ArrayScan VT1 High Content Analysis Reader (Thermo Scientific) and the images were analyzed by SpotDetectorV3 BioApplication software. CXCR4 inhibitory activity of AMD3100 was set as 100% and CXCR4 inhibitory activity in cells treated with SDF-1α only was set as 0%. CXCR4 antagonistic activity of the polyplexes is expressed as mean % inhibition ± SD (n = 4).

2.7. Flow cytometry

CD18/HPAF.luc cells were seeded in 6-well plates in DMEM with 10% FBS. The next day, fresh DMEM with or without 10 µM gemcitabine was added. After 24 h of incubation, cells were washed with PBS twice, detached using enzyme-free PBS-based cell dissociation buffer (Gibco by Life Technologies, Grand Island, NY), and resuspended in PBS. After centrifugation at 800 rpm for 5 min, the supernatant was removed and the cells were resuspended in FACS buffer (1% BSA, 0.1% NaN₃ in PBS) to obtain final cell concentration of 5×10^6 /mL. The cell suspension (100 µL) was mixed with 20 µL of APC mouse anti-human CXCR4 antibody or the corresponding isotype control. After incubation at room temperature for 30 min, the cells were washed three times with FACS buffer to remove free antibody and resuspended in 500 µL of FACS buffer for flow cytometry. The data were processed and analyzed using FlowJo software (Tree Star Inc., Ashland, OR) and reported as percentage of CXCR4-positive cells.

2.8. Western blotting

To determine total cellular levels of protein, whole-cell lysate was prepared in Pierce® RIPA lysis buffer (Thermo Scientific, Rockford, IL) supplemented with 1x protease and phosphatase inhibitor cocktail. Total protein content was quantified using BCA assay, and samples were normalized by dilution with RIPA buffer to obtain equal protein concentration. Equal volume of 2x Laemmli sample buffer (BIO-RAD, Hercules, CA) was added, followed by boiling in water for 5 min. Equal amounts of total protein (20-40 µg) were loaded to SDS-polyacrylamide electrophoresis gel, run first at 80 V for 30 min and then at 120 V for 2 h. The protein contents were transferred to polyvinylidene difluoride (PVDF) membrane, and the membrane was blocked with 5% skim milk at room temperature for 1 h, and incubated overnight with the primary antibodies at 4 °C. Next day, the blot was washed three times with TBST buffer (20 mM Tris, 500 mM NaCl, 0.1% Tween 20, pH 7.5) and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The protein signals were visualized by Pierce® ECL Western blotting substrate (Thermo Scientific, Rockford, IL). The band intensity was evaluated using ImageJ software.

2.9. RNA isolation and Real Time PCR analysis

The total RNA from the cells was isolated using TRIzol[®] reagent and the RNA from tissues was isolated using mirVana miRNA isolation kit according to the protocols from Life Technologies. Cells were washed with PBS and lysed in the TRIzol[®] Reagent. The homogenized samples were incubated at room temperature for 5 min to complete dissociation of the nucleoprotein complex. Chloroform was added and the samples were centrifuged. RNA was precipitated by addition of 0.5 mL of isopropanol to the aqueous phase. RNA pellet was resuspended in RNase-free water and stored at -80°C . For isolation of RNA from tissues, tumor tissues were homogenized in liquid nitrogen and lysed in the lysis/binding buffer. miRNA Homogenate Additive was added to the tissue lysates and acid-phenol: chloroform was added and vortexed for 1 min. The aqueous phase was collected, mixed with equal volume of ethanol, and the mixture was passed through a filter cartridge. The RNA isolated from cells and tissues was reverse-transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA) using a thermal cycler in Rotor-Gene Q RT-PCR (Qiagen, Hilden, Germany). The thermal cycling conditions used were 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. The expression pattern of the gene of interest was analyzed and the fold change in gene expression was calculated using the $2^{-\text{CT}}$ method. Results were expressed as mean expression compared with untreated cells $\pm$ SD ($n=3$) and Student's t-test was used to determine the statistical significance (p value less than 0.05 was considered as significant).

2.10. Cytotoxicity analysis

PCX cytotoxicity was evaluated by CellTiter-Blue Cell Viability Assay (Promega, Madison, WI) in CD18/HAPF.luc and HepG2 cells. Single cell suspension was seeded in 96-well plates. After overnight incubation, culture medium was replaced with 150 μL of serial dilutions of a polymer in serum-supplemented medium. Cells were incubated at 37°C for 24 h, and the medium was replaced with a mixture of 100 μL medium and 20 μL of CellTiter-Blue reagent. After 1 h incubation, the fluorescence intensity [I] was measured by SpectraMax[®]M5e Multi-Mode Microplate Reader at $\lambda_{\text{ex}}/\lambda_{\text{em}}$ 560/590 nm. The relative cell viability (%) was determined as $[\text{I}]_{\text{sample}}/[\text{I}]_{\text{untreated}} \times 100\%$. The IC₅₀ was defined as the polymer concentration that causes 50% decrease in cell viability relative to untreated cells ($n=3$) and calculated in GraphPad Prism using a built-in dose-response analysis.

2.11. Cell migration analysis

CD18/HAPF.luc cells were treated with 1 or 10 μM gemcitabine for 24 h, trypsinized, and resuspended with 1 $\mu\text{g}/\text{mL}$ PCX in serum-free medium for 15 min. The cell suspension with PCX was then added to transwell inserts using 200,000 cells in 300 μL medium per insert. DMEM with 10% of FBS was added as the chemoattractant in the companion plate. After 24 h, cotton swabs were used to remove the non-invaded cells on the upper surface of the insert membrane. The invaded cells on the bottom surface were fixed in 100% methanol for 10 min at room temperature and followed by staining with 0.2% Crystal Violet solution for 10 min at room temperature. The migrated cells were counted at $10\times$ magnification. The results were expressed as percentage of migrated cells relative to untreated cells/imaging area $\pm$ SD ($n = 4$ random imaging areas of triplicate samples).

2.12. Orthotopic implantation of tumor cells and analysis of anticancer activity

All described procedures were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee. Orthotopic implantation of pancreatic cancer cells was performed as described previously [36]. Briefly, CD18/HPAF.luc cells were trypsinized, washed and resuspended in sterile PBS. Female athymic nude mice (6 weeks old) purchased from Harlan Laboratories were anesthetized with 350 μ L of intraperitoneal injection of a 4:1 mixture of ketamine (100 mg/mL) and xylazine (20 mg/mL) diluted 10 times in sterile water. The surgical site was sterilized and a 1-cm incision was made in the peritoneum at the mid-abdomen region below the sternum by scissors. Total of 2.5×10^5 of CD18/HPAF.luc cells were injected into the head of pancreas without causing injury and torsion. The abdomen was closed using a 2-layer suture with 5-0 chromic catgut and soft staple. The skin staples were removed 10 days after surgery. After 10 days of growth, the luciferase-expressing tumors were imaged by IVIS and the mice were randomly assigned into three groups: saline (n=7), negative siRNA control polyplexes (Chol17-siScr/PCX, n=7) and combination polyplexes (Chol17-siNCOA3/PCX, n=6) group. The PCX/siRNA polyplexes were prepared at w/w 2 and administered 3 times per week through tail vein (40 μ g siRNA/mouse). The mice were observed and weighed every other day. Tumor growth and total tumor burden were monitored by palpation and whole-body IVIS bioluminescence imaging. Mice were sacrificed after 39 days of tumor growth. Changes in tumor growth and sites of metastasis were evaluated in each experimental group. Pancreatic tumors from different experimental groups were collected for immunohistochemistry (IHC) analysis.

2.13. Immunohistochemistry analysis (IHC)

For histopathological analysis, the tumor tissues were fixed in 10% formalin for 72 h and stored in 75% ethanol. The tissues were embedded in paraffin and 5 μ m sections were cut and stained with haematoxylin & eosin (H&E) as well as requisite antibodies as described previously [7]. Briefly, tissue slides were baked overnight at 58 °C. Next day, tissues were deparaffinized, hydrated and antigens were recovered by boiling in citrate buffer. Tissue sections were blocked with 2.5% horse serum and incubated with indicated primary antibody (8G7) overnight at 40 °C. Tissues were washed and incubated with HRP-conjugated secondary antibody. Subsequently, tissues slides were washed and developed using 3,3'-diaminobenzidine kit (Vector Laboratories, Burlingame, CA) for colorimetric detection and counterstained with hematoxylin. Tissues were dehydrated, dried and mounted with Permount and evaluated by a pathologist.

2.14. Tumor perfusion analysis

Mice were evaluated at the end of the treatment (before euthanasia) by magnetic resonance imaging (MRI) to assess tumor vascular function using perfusion imaging, after acquiring a reference T₂-weighted image set to visualize the tumor location. Mice were anesthetized by inhalation anesthesia (1.5% isoflurane). The anesthetized mice were positioned in a Plexiglas holder and placed in an RF coil for imaging. The anesthetized animal was secured in place to a support platform upon which the animal's body rested horizontally for MRI acquisition. The breathing rate and temperature of the anesthetized animals were monitored continuously with this holder design using an SA Instruments (Stony Brook, NY) model

1025 small animal monitoring and gating system. During image acquisition, animals were maintained on 0.5-1.5% isoflurane, in oxygen with an output of 1 L/min with gases continuously vacuumed from the opposite side of the chamber using the facility vacuum line regulated at a flow rate of 1 L/min. Animals were monitored during the procedure and respiration rates were maintained at 40-60 bpm by adjusting isoflurane during examinations. The holder with the mouse was inserted into a birdcage quadrature transmit/receive coil and mounted at the magnet center. Studies began with a localizer to center the head in the magnet and coil. After localization and system shimming, T₂-weighted high-resolution anatomical image was obtained for each animal, followed by the acquisition of perfusion maps. Perfusion maps were acquired and analyzed using Flow Sensitive Alternating Inversion Recovery (FAIR) [37]. Perfusion maps were generated with a Rapid Acquisition with Relaxation Enhancement (RARE) readout. Images were acquired with a RARE factor of 16, 16 inversion recovery times ranging from 30-2300 ms, 1 mm slice thickness, selective inversion slab thickness of 4 mm, 30 mm × 30 mm field of view, 128 × 128 matrix. Total imaging time for MRI was 0.5-1 hour per animal.

3. RESULTS AND DISCUSSION

We have previously reported the synthesis of polycations with an ability to simultaneously inhibit the chemokine receptor CXCR4, and deliver nucleic acids to the cells. We have successfully used these polymeric CXCR4 inhibitors (PCX) to deliver plasmid DNA, siRNA, and microRNA *in vitro* [30-33]. We have subsequently confirmed antimetastatic potential of the PCX polymers without therapeutic nucleic acids in an experimental lung metastasis model of melanoma [38]. In the present study, we describe for the first time the application of PCX as dual function systems capable of systemic delivery of therapeutic siRNA for the treatment of metastatic PC.

3.1. PCX selection

Delivery of siRNA by polyplexes requires polymers with the ability to electrostatically bind and protect the siRNA. All the PCX polymers used in this study are poly(amido amine)s, synthesized by Michael-type polyaddition of cyclam-based CXCR4 antagonists [30, 32, 33]. The synthesis and characterization of all the PCX were reported in our previous publications, but the polymers were never compared simultaneously. With the goal of selecting PCX with the most favorable properties for the present study, we have chosen two unmodified PCX (Figure 1): (i) branched PCX-1 synthesized from a commercial CXCR4 antagonist AMD3100 [32] and (ii) linear PCX-2 synthesized from a monocyclam antagonist monomer developed in our lab [33]. We have also selected two PCX-1, covalently modified with 17 wt% and 25 wt% cholesterol (Chol17-PCX and Chol25-PCX) due to their previously reported high siRNA delivery efficiency and improved polyplex stability against disassembly [30]. Lastly, we have selected PEGylated PCX-1 (PEG-PCX) to prepare siRNA polyplexes with improved colloidal stability and reduced positive surface charge [31].

Toxicity of polycations remains a major concern for their application in nucleic acid delivery [23]. Polycation toxicity is known to correlate with several key factors including molecular weight, charge density, chemical structure, and biodegradability [39-40]. We first compared

cytotoxicity of all PCX in HepG2 cells often used to gauge potential hepatotoxicity of novel compounds (Figure 2a). All PCX were significantly less toxic compared to benchmark poly(ethylene imine) (PEI) used as a control. Not surprisingly, PEG-PCX showed the lowest toxicity of the polycations based on PCX-1. The decreased cytotoxicity is a direct result of steric hindrance of PEG that prevents interaction of the polycations with cell membranes and vital intracellular proteins [41–42]. The modification of PCX-1 with cholesterol had no adverse effect on cytotoxicity in HepG2 cells, suggesting that despite the enhanced hydrophobicity of PCX, this modification would not cause cell membrane disruption. As expected, linear PCX-2, which had lower molecular weight and charge density than the branched PCX-1, proved to be the least toxic of all the tested polymers. We then determined cytotoxicity of PCX in the CD18/HPAF.luc cell line to establish safe concentration for the subsequent *in vitro* optimization studies. Similar to HepG2 cells, all PCX were less toxic than PEI in the CD18/HPAF.luc cells, although the PC cells showed enhanced sensitivity to the toxic effects of the PCX and the differences between PCX and PEI were less pronounced. While the reasons for increased sensitivity of the PC cells to PCX are not fully understood at this point, such preferential cytotoxicity when compared with PEI may be beneficial in improving anticancer activity of the PCX-based systems.

3.2. Characterization of PCX/siNCOA3 polyplexes

The ability of PCX to form polyplexes with siNCOA3 was investigated by electrophoresis. The polyplexes were prepared at increasing PCX-to-siNCOA3 w/w ratios. The w/w ratios are expressed without taking into account cholesterol or PEG content in the copolymers. As shown in Figure 2b, all PCX were able to form complexes with siNCOA3 at or above w/w ratio of one, as indicated by the absence of free siRNA in the gel. The presence of a smear in PEG-PCX and Chol25-PCX samples suggested weaker complexation ability of these two copolymers, when compared to the other PCX tested. To assure complete siRNA complexation with the tested polycations, all polyplexes used in this study were prepared at w/w ratio of 2.

Hydrodynamic size and zeta potential of PCX/siNCOA3 polyplexes were measured by dynamic light scattering (Figure 2c). Polyplexes were prepared in HEPES-buffered glucose (HBG) at w/w ratio of 2 and incubated for 20 min at room temperature before measurement. With the exception of PCX-2/siNCOA3, all other polyplexes exhibited small particle sizes ranging from ~90 to 125 nm. The presence of cholesterol did not result in any significant aggregation as suggested by comparable or smaller sizes of Chol-PCX/siNCOA3 particles compared to control PCX-1/siNCOA3. All PCX formed polyplexes with a positive surface charge and zeta potentials ranging from 5 to 23 mV. As expected, PEG-PCX polyplexes showed nearly neutral zeta potential because of the shielding effect of PEG.⁴⁰

The ability to inhibit CXCR4 is critical part of the proposed mechanism of action of the PCX polyplexes (Scheme 1). We compared the ability of the different PCX/siRNA polyplexes to inhibit CXCR4 using a phenotypic receptor redistribution assay (Figure 3) [33]. The assay determines the ability of PCX to inhibit redistribution of EGFP-conjugated CXCR4 receptor from plasma membrane (diffuse green fluorescence in Figure 3b) to lysosomal compartment (punctate fluorescence). We also used negative control siRNA

(siScr) to prepare the polyplexes to avoid any potential confounding effects from NCOA3 silencing. As shown in Figure 3b, the control small-molecule CXCR4 antagonist AMD3100 inhibits EGFP-CXCR4 internalization, as indicated by the diffuse pattern of fluorescence. However, the untreated cells display punctate fluorescence indicative of EGFP-CXCR4 internalization into endosomes. Activity of PCX was analyzed in this assay and expressed as % CXCR4 antagonism relative to the control AMD3100. All PCX polyplexes exhibited nearly complete CXCR4 inhibition when prepared at w/w ratio of 2. Unmodified PCX-1 and PCX-2 were the most effective inhibitors, together with PEG-PCX polyplexes. Modification with cholesterol decreased the inhibitory activity of the polyplexes, but even the least active Chol25-PCX/siScr showed 91% CXCR4 inhibition. In contrast, PEI polyplexes had no discernible activity against CXCR4, confirming that the observed effect is not due to polycationic character of PCX but due to its specific binding to the CXCR4 receptor.

3.3. NCOA3 knockdown by PCX/siNCOA3 polyplexes

After confirming the ability of PCX to form polyplexes with siRNA and to maintain strong CXCR4 antagonism, we evaluated PCX ability to deliver siNCOA3 and to downregulate NCOA3 gene in PC cells. The polyplexes were prepared at w/w ratio of 2 using either siNCOA3 or a negative control siRNA (siScr). PEI/siRNA polyplexes prepared at w/w of 1.5 were used as controls. As shown in Figure 4, we observed large differences in the ability of the tested polyplexes to silence the NCOA3 expression. Both Chol-PCX/siNCOA3 polyplexes exhibited significantly higher NCOA3 silencing when compared to the other tested polyplexes. Chol17-PCX proved to be the most efficient as it achieved nearly 80% NCOA3 gene silencing. Although control PEI demonstrated second best gene silencing (59%), it could only achieve such activity with significant off-target effects as demonstrated by decreased NCOA3 levels observed even with PEI/siScr [43]. PCX-1 and PCX-2 showed only marginal gene silencing activity and PEG-PCX had nearly no silencing effect.

3.4. Inhibition of PC cell migration by PCX

Multiple studies have demonstrated that in addition to promoting cancer cell proliferation, CXCR4 overexpression increases PC cell motility and invasion, which leads to enhanced metastasis [10, 44, 45]. There is a growing experimental and clinical evidence that anticancer therapies that promote hypoxic environment may increase CXCR4 expression and thus inadvertently enhance the metastatic potential of the tumors [46-48]. Even gemcitabine, the standard of care for PC, has recently been shown to upregulate CXCR4 expression in multiple PC cell lines, lending further support to the notion that some current treatments may be promoting aggressive PC phenotype [49, 50]. The gemcitabine-induced upregulation of CXCR4 was previously reported in Colo357 and MiaPaCa PC cell lines [49]. Here, we determined if gemcitabine had similar effect in the CD18/HPAF.luc cells used as PC model in the present study. The cells were treated with 1 and 10 μ M gemcitabine and expression of CXCR4 was measured by flow cytometry and Western blot analysis. As shown in Figure 5a, gemcitabine treatment increased the population of CXCR4-positive CD18/HPAF.luc cells from 11.3% to 18%. Western blot analysis corroborated this finding, as there was a noticeable increase in CXCR4 expression in CD18/HPAF.luc cells at both 1 and 10 μ M gemcitabine concentrations (Figure 5b).

Having confirmed that gemcitabine treatment increased the CXCR4 expression in CD18/HPAF.luc cells, we tested the ability of PCX to inhibit CXCR4-mediated migration of these cells. CXCR4 antagonists can inhibit migration of cancer cells that utilize the CXCR4/SDF1 axis and we have previously shown that PCX and their polyplexes inhibited CXCR4-mediated migration and invasion in human osteosarcoma and cholangiocarcinoma cells [30, 33, 51]. Here, we studied the migration of gemcitabine-treated CD18/HPAF.luc cells in a Boyden chamber using 10% serum as the chemotactic signal. As expected, the cells readily migrated toward the chemotactic gradient (Figure 6). No significant migration was observed in CD18/HPAF.luc cells without gemcitabine treatment (not shown). All tested PCX used at 1 µg/mL were able to inhibit cell migration. The observed inhibition ranged from 38% for Chol25-PCX to 43% for PCX-2. In comparison, small-molecule CXCR4 antagonist AMD3100 was able to reduce the number of migrated cells by only 29.5% and PEI had no effect on cell migration. This finding supports the use of PCX as a novel antimetastatic PC therapy because of more efficient prevention or delay of PC metastasis than conventional CXCR4 antagonists.

3.5. Inhibition of primary pancreatic tumor growth by PCX/siNCOA3 polyplexes

Both CXCR4 and NCOA3 are involved in the proliferation of PC cells and in maintenance of tumor microenvironment that promotes metastatic spread. Although we were expecting antimetastatic activity of PCX/siNCOA3, we first tested if combining inhibition of CXCR4 and NCOA3 also affects the growth of the primary PC tumors. To test this, CD18/HPAF.luc cells were orthotopically implanted into the pancreas of female nude mice and allowed to grow for 10 days (Figure 7). The body weight of mice decreased less than 10% after tumor cell implantation, however, all of the mice recovered after 7 days (Figure 7a).

When the presence of the tumors was established by whole-body bioluminescence imaging on day 10, the mice were randomized into three experimental groups and treated *via* tail vein injections with polyplexes prepared at a polycation/siRNA w/w ratio of 2. Control animals were treated with saline. Despite aggressive administration regimen to maximize the therapeutic effect, none of the treatments resulted in any significant effect on the body weight, suggesting no major toxicity associated with the use of PCX/siNCOA3 polyplexes. The growth of the primary PC tumors was monitored by bioluminescence imaging (Figure 7b) and the mice were sacrificed on day 39 post-implantation. As shown in Figure 7b-d, treatment with both the PCX/siNCOA3 as well as PCX/siScr significantly slowed down the growth of the primary pancreatic tumor. Superior activity of PCX/siNCOA3 when compared with PCX/siScr suggested the benefits of combined inhibition of CXCR4 and NCOA3 silencing on the progression of primary PC tumors. The NCOA3 gene silencing at the end of the experiment was confirmed by qRT-PCR (Figure 7e).

3.6. Antimetastatic effect of PCX/siNCOA3 polyplexes

The most common site of distant metastasis in PC patients is the liver [52]. However, PC can metastasize to broad range of organs and tissues, including diaphragm, gallbladder, small and large intestines, ovaries, pericardium, stomach, and spleen [52, 53]. As expected, untreated animals presented with widespread metastatic disease on necropsy at day 39 (Figure 8). All untreated animals showed metastasis in the stomach and ovaries, 6 out of 7 had

metastasis in the diaphragm and small intestine, and 5 out of 7 had the disease spread to the liver, spleen, and mesenteric lymph nodes.

Treatment with PCX/siNCOA3 resulted in greatly reduced incidence of metastases in all organs and tissues. Importantly, we observed no metastasis in the liver, stomach, and ovaries of the treated animals. In most organs, the PCX/siNCOA3 treatment showed better antimetastatic effect than PCX/siScr. The comparison between PCX/siNCOA3, which inhibits both CXCR4 and NCOA3 and control PCX/siScr, which only inhibits CXCR4 reveals the important contribution of NCOA3 silencing to the overall antimetastatic activity of the polyplexes. The finding that CXCR4 inhibition by PCX exerts its strong antimetastatic effect in some organs but not in others is a reflection of likely involvement of other chemokine receptors in the metastasis to those organs. Our previous study has shown that NCOA3 upregulates the expression of fucosyltransferase 8 (FUT8) in PC cells. Fucosyltransferase expression in PC is associated with the increased expression of sialyl-Lewis a/x on the cancer cells [54], which act as the ligands for the selectins, present on the surface of endothelial cells, therefore, facilitating the interaction between cancer and endothelial cells during extravasation step of metastasis [54]. Further, fucosylation is also associated with the stem cell-like phenotype and the malignant transformation of intraductal papillary mucinous lesions [55].

3.7. Regulation of mucin expression and tumor perfusion by PCX/siNCOA3 polyplexes

As postulated from previous study, NCOA3 may participate in the hardening of the desmoplasia by upregulating the expression of LOXL2, which promotes hypoxic environment leading to necrosis. Our previous study has also demonstrated that the silencing of NCOA3 specifically targets the pathways involved in stellate cell activation and upregulation of chemokine and extracellular matrix proteins. H&E staining in Figure 9a revealed the inner structure of the primary PC orthotopic tumors. The pink part corresponds to the tumor necrosis, which was induced by the hypoxia in the PC tumor microenvironment. We observed a significant decrease in the necrotic area from ~17% to 2.5% after PCX/siNCOA3 treatment compared to PCX/siScr ($p = 0.012$) and untreated controls (Figure 9b).

Immunohistochemistry analysis further demonstrated decreased MUC4 expression in primary PC tumors by PCX/siNCOA3 in comparison with both the untreated and PCX/siScr control groups. Poor tumor perfusion is one of the most critical barrier for the effective delivery of the chemotherapeutics to the pancreatic tumor bed. We expected to enhance the tumor perfusion after NCOA3 inhibition due to its role in activation of pancreatic stellate cells and desmoplastic reaction (Scheme 1). Other studies targeting LOXL2 have also demonstrated the survival advantage due to reduced desmoplasia and extracellular matrix [56, 57]. To support the positive role of the PCX/siNCOA3 treatment in overcoming PC desmoplasia, tumor perfusion was evaluated by magnetic resonance imaging (Figure 9c). There was noticeable increase in the tumor perfusion from 44 to 62 mL/100 g/min following treatment with PCX/siNCOA3. The increased tumor perfusion after administration of the dual function polyplexes is important, as it will aid in ameliorating the hypoxic microenvironment of PC, often associated with aggressiveness and chemoresistance of PC.

4. CONCLUSION

We developed dual-function polyplexes to simultaneously deliver siNCOA3 and inhibit CXCR4 chemokine receptor to treat metastatic PC. Our study showed that cholesterol modification of the polymeric CXCR4 antagonists provided the most favorable set of properties to achieve efficient gene silencing, CXCR4 antagonism, and physicochemical properties for siRNA delivery to PC. NCOA3 plays important role in multiple processes that maintain supportive pro-inflammatory tumor microenvironment in PC. Our study is the first to report NCOA3 as a suitable target for nanomedicine strategies that seek to modulate PC tumor microenvironment to decrease desmoplasia as a way of enhancing drug delivery and overcoming chemoresistance. Such changes to the tumor microenvironment that lead to enhanced tumor perfusion, however, may also inadvertently promote metastasis. Thus, combining tumor-microenvironment modulating strategies, with simultaneous antimetastatic ability of CXCR4-inhibiting polymers, promises to minimize such adverse effects. This notion is clearly supported by the strong antimetastatic effect achieved with the PCX/siNCOA3 polyplexes. Since desmoplasia and CXCR4 overexpression are characteristic features of both primary and metastatic PC tumors, the developed polyplexes are suitable for delivery to both primary and metastatic PC sites [58]. Despite tremendous promise in anticancer therapies, siRNA is most likely to be used in combination with other treatments. Our study lays strong foundation and support for the use and development of PCX/siNCOA3 as neoadjuvant or adjuvant treatment in combination with conventional chemotherapeutics such as gemcitabine. In particular, given the well-established fact that gemcitabine treatment promotes metastatic PC phenotype by upregulating CXCR4 expression, the use of PCX/siNCOA3 represents strong rationale for such approach.

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Figure 1.
Chemical structures of polymeric CXCR4 inhibitors PCX-1 and PCX-2.

(a) Cytotoxicity of PCX (IC₅₀ in $\mu\text{g/mL}$)

Cell line	PCX-1	Chol17-PCX	Chol25-PCX	PCX-2	PEG-PCX	PEI
HepG2	52.3	47.9	59.6	186	144	25.1
CD18/HPAF.luc	16.3	25.8	44.5	25.4	22.2	11.1

Figure 2.

Characterization of PCX and PCX/siNCOA3 polyplexes. (a) Cytotoxicity of PCX in HepG2 and CD18/HPAF.luc cells. IC₅₀ (in $\mu\text{g/mL}$) were calculated as the polymer concentration that achieves 50% decrease in cell viability relative to untreated cells ($n = 3$). (b) Ability of PCX to form polyplexes with siNCOA3 evaluated by agarose gel retardation assay at increasing PCX/siNCOA3 w/w ratios. (c) Hydrodynamic size and zeta potential of PCX/siNCOA3 polyplexes prepared at w/w 2 (mean $\pm$ SD, $n = 3$). All polyplexes were prepared in HBG at siNCOA3 concentration of 20 $\mu\text{g/mL}$.

Figure 3.

CXCR4 antagonism of PCX/siScr polyplexes (polymer = 1 μ g/mL, w/w 2) in U2OS cells.

(a) The results are shown as mean % CXCR4 inhibition relative to AMD3100 $\pm$ SD (n=4).

One-way ANOVA with Tukey-Kramer multiple comparisons test (***)P<0.001 vs. PEI).

(b) Representative images of EGFP-CXCR4 distribution in cells treated with different polyplexes. The scale bar is 200 μ m.

Figure 4.

NCOA3 gene silencing by PCX/siNCOA3 polyplexes in CD18/HPAF.luc PC cells. NCOA3 silencing was determined by Western blot (top) using polyplexes prepared at w/w ratio of 2 and used at 200 nM siRNA. The percent of NCOA3 silencing (bottom) was calculated from NCOA3 band intensity of PCX/siNCOA3 relative to the corresponding PCX/siScr control. PEI polyplexes were prepared at w/w 1.5.

Figure 5.

Effect of gemcitabine (Gem) on the CXCR4 expression in CD18/HPAF.luc PC cells. (a) Cell surface CXCR4 expression in CD18/HPAF.luc cells by flow cytometry before and after gemcitabine treatment. (b) Total cellular CXCR4 expression by Western blot (n=2). Results are expressed as ratio of a mean relative CXCR4 expression vs. cells not treated with gemcitabine (n=2).

Figure 6.

Inhibition of PC cell migration by PCX (1 μg/μL). CD18/HPAF.luc cells were treated with 10 μM gemcitabine for 24 h before the migration study. Migrated cells were counted in 4 randomly selected imaging areas at 10x magnification of triplicate samples (scale bar = 400 μm). One-way ANOVA with Tukey-Kramer multiple comparisons test (***P<0.001, vs. untreated cells).

Figure 7.

Effect of systemic treatment with Chol17-PCX/siNCOA3 (w/w 2) on the growth of primary pancreatic tumor. (a) Average body weight after orthotopic implantation of CD18/HPAF.luc cells and during treatment with Chol17-PCX/siNCOA3 (n=6), Chol17-PCX/siScr (n=7), and saline (n=7). (b) Primary tumor growth evaluated by whole-body bioluminescence imaging. (c) Primary tumor weights were measured after necropsy. (d) Photograph of resected primary PC tumors. (e) Silencing of NCOA3 expression in the primary PC tumors by RT-PCR (results shown as ratio of mean relative NCOA3 expression compared with saline treated mice $\pm$ SD (n=3)). Statistical comparisons by unpaired t-test (***p<0.001, **p<0.01, *p<0.05).

Figure 8.

Effect of PCX/siNCOA3 treatment on the incidence of PC metastasis in the orthotopic CD18/HPAF.luc PC model. The presence of metastasis in major organs and tissues was determined on day 39 following implantation of the PC cells in mice.

Figure 9. Analysis of primary pancreatic tumors after treatment with Chol17-PCX/siNCOA3 polyplexes. (a) H&E staining and MUC4 immunohistochemistry analysis (magnification 40x). (b) Extent of necrosis in primary tumor determined from the H&E staining. (c) Primary tumor perfusion on day 39 determined from magnetic resonance imaging.

Scheme 1.

Proposed mechanism of action of PCX/siNCOA3 polyplexes.