Non-viral siRNA delivery systems for pancreatic cancer therapy

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Abstract

The serious drawbacks of conventional pancreatic ductal adenocarcinoma (PDAC) therapy like nonspecific toxicity and high resistance to chemo and radiation therapy, prompted the development and application of countless siRNA-based therapeutics. Significant technological success has been achieved in this area; however, the major challenges to siRNA-based therapeutics becoming a new paradigm in the pancreatic cancer therapy stem from enzymatic digestion, off-target effects, difficulty to enter cells, induction of innate immune responses, and renal clearance. Recent advances in drug delivery systems hold great promise for improving siRNA-based therapeutics and developing a new class of drugs, nano-siRNA drugs. However, a number of fundamental questions, regarding toxicity, immunostimulation, and poor knowledge of nano-bio interactions, need to be addressed before clinical translation. In this review, we provide recent achievements in designing and development of various non-viral delivery vehicles for pancreatic cancer therapy. More importantly, co-delivery of conventional anticancer drugs with siRNA as a new revolutionary pancreatic cancer combinational therapy is completely discussed.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) remains as one of the leading causes of global cancer-related death with a median survival of six months after diagnosis (Ilic & Ilic, 2016; Kamisawa, Wood, Itoi, & Takaori, 2016). Depending on the stage and patterns of tumor growth and patient characteristics, different treatment strategies such as radiation therapy (RT), chemotherapy (alone or in combination) and surgery have been developed for the pancreatic tumor therapy (Kami et al., 2005; Kleeff et al., 2016). Adjuvant chemotherapy after surgical resection is the treatment of choice for the early stages of the disease. In spite of progress in the detecting, managing, and treating techniques of pancreatic cancer, the five-year survival rate only reaches to around 9%. Two combination chemotherapy regimens including 5-fluorouracil (5-FU)/leucovorin with irinotecan and oxaliplatin (FOLFIRINOX), and gemcitabine (GEM) with nab-paclitaxel are currently the gold standard treatments for fmametastatic pancreatic cancer. Although these strategies improved the prognosis of advanced pancreatic cancer, short half-life of drugs in blood, non-specific toxicity, and multidrug resistance (MDR) still cause poor clinical outcomes (Cives & Strosberg, 2018; Wolfgang et al., 2013).

In recent years, siRNA-based therapeutics have emerged as revolutionary therapeutic modalities for the treatment of human diseases, especially malignant cancers, by selectively targeting disease-causing genes. Furthermore, the combination of siRNA-based therapeutic agents with conventional anti-cancer drugs can represent a new powerful strategy to overcome MDR and pancreatic cancer (Oh & Park, 2009). Despite its great potential, *In vivo* siRNA-based therapy faces major challenges including poor cellular uptake, off-target effects, enzymatic degradation, immune recognition, and rapid clearance (C. I. E. Smith & Zain, 2019).

To address these limitations, nanoparticle-based carriers make the targeted delivery of siRNAs and chemotherapeutic agents possible through effective and safe means (Shahin Aghamiri, Keyvan Fallah Mehrjardi, et al., 2019). Because of their enhanced permeability and retention (EPR), these carriers are potential choices for delivery of poorly soluble medications, encapsulation and preferential accumulation and concentration of drug-loaded nanocarriers in the tumor cells (Riley, June, Langer, & Mitchell, 2019). Altogether, nanoparticle-based delivery, chemotherapy, and siRNAs are practical strategies that can represent an exciting potential class of therapeutic agents to resensitize pancreatic tumor cells and facilitate pancreatic tumor therapy. Here, we present an overview of new achievements and limitations in designing novel nanocarrier-based therapeutic approaches for the treatment pancreatic malignancies. Moreover, proposed methods for the delivery of chemotherapy drugs and/or imaging agents with siRNA for synergistic anti-pancreatic cancer properties will be reviewed.

Overview of delivery of siRNA with nanocarriers for pancreatic cancer therapy

RNA interference (RNAi) mechanism in mammalian cells has brought new research areas for the treatment of numerous diseases. Small interfering RNA (siRNA) is a duplex RNA consisting of 21-23 nucleotides, which is responsible for RNAi-based gene silencing (Davis et al., 2010). When siRNA is produced by Dicer processing of long double-stranded RNAs or synthetic siRNAs are delivered into the cytoplasm. Subsequently, siRNA binds to the RNA-induced silencing complex (RISC). This complex is the platform on which the transformation of double strands to single-stranded by Argonaute-2 takes place and one strand mitigates a sequence-specific recognition of mRNA. The activated RISC recognizes the target transcript based on the sequence homology. Consequently, degradation starts from the 5' end of antisense strand at the opposite of position 10 (B. Kim, Park, & Sailor, 2019).

In the past decade, the therapeutic potentials of siRNA have been proven in the treatment of genetic diseases, virus infections, and cancers (Sousa, Oliveira, Oliveira, & Sarmento, 2019). By designing the sequence of siRNA, any genes playing important role in the development of various diseases can be targeted in theory, including previously undruggable targets. Furthermore, various disorders such as viral infections, hereditary diseases, and tumors may benefit from its therapeutic potential.

In spite of the great potentials of siRNA to be developed as a drug, there are serious limitations that are hindering their practical applications. For instance, siRNAs do not simply cross over cytomembrane because of their low molecular weight (13 kDa) and anionic net charges. Moreover, siRNAs are susceptible to RNase digestion and rapid excretion through renal system. Furthermore, the accumulation of siRNAs in tumor cells is very low (Onoue, Yamada, & Chan, 2014). To address these challenges, siRNA chemical modifications and designing of innovative nanocarriers for delivery of siRNA have offered new opportunities for pancreatic cancer nano-siRNA drug development (Table 1). In this manner, siRNA-conjugated nanocarriers with a higher molecular weight can delay renal clearance and increase the accumulation of siRNAs in cancer cells (P. Zhang et al., 2018). Therefore, various nano-siRNA therapeutic agents have been designed and employed for clinical trials (Table 2). In this part, nanoparticle-based systems using lipid, rigid-particle, polymer, and specific ligands for the treatment of pancreatic cancer will be described in detail (Figure 1).

Figure 1. Instances of nanocarriers for pancreatic cancer therapy.

Biological barriers to delivery of siRNA with nanocarriers

Extracellular barriers

Effective siRNA delivery is initially disrupted by the hostile extracellular environment including all chemical, biological, and physical barriers, like the immune system reactions, scavengers, nucleases and proteases together with extreme pH (Hill, Chen, Chen, Pfeifer, & Jones, 2016). Kidney glomerulus is a critical physically filtrating challenge in siRNA delivery in which molecules with small size and water are rapidly cleared into urine whereas molecules with higher molecular weight are retained in the bloodstream (Choi et al., 2007). The pore diameter of the glomerular basement membrane (GBM), a thin (250–400 nm) non-cellular layer of the glomerular filtration barrier, is demonstrated to be around 6–10 nm. Hence, siRNAs without any delivery systems due to their small size (i.e. about 7.5 nm in length and 2 nm in diameter) can be easily filtered within 10 min via GBM (Abedini, Ebrahimi, & Hosseinkhani, 2018). Therefore, it is necessary to set a lower size limitation of about 10 nm for the delivery systems design (Lu & Qiao, 2018). By the way, the defective "leaky" vascular architecture of several solid tumors, which is related to immature lymphatic ducts, allow size-dependent accumulation of nanoparticles (10-100nm) in tumor. This preferential size-dependent accumulation of drug-loaded nanocarriers in the cancer cells was initially called the enhanced permeability and retention (EPR) effect in 1986 (Davoodi et al., 2018; Kalyane et al., 2019). To date, the EPR phenomenon has been broadly established in different mice models of pancreatic tumors using various types of delivery vehicles (Aghamiri, Jafarpour, & Shoja, 2019). It is noteworthy that this phenomenon is strongly affected by pancreatic tumor physiology. While highly permeable LS174T-transplanted SCID model mice are reported to permit substantial accumulation of even 400 nm nanoparticles (Yuan et al., 1995), mice bearing BxPC3 tumors demonstrated hypervascularity and thick fibrotic stroma impeding tumor accumulation of >50 nm-sized nanoparticles; unlike 30 nm-sized ones (H. Cabral et al., 2011). Finally, high antitumor activity offers intriguing glimpses into the potential of 30 nm-sized nanoparticles in pancreatic cancer therapy (Horacio Cabral et al., 2013). As a consequence, many nanoparticle-based delivery systems with a size of less than 50 nm have been designed for increased accumulation in pancreatic tumor tissues (Maeda, 2015).

Because of high cytidine deaminase (CDA) expression as well as physical blockage, stroma can contribute to pancreatic cancer chemoresistance and unfavorable pharmacokinetics and pharmacodynamic profile *in vivo* , which can decrease the systemic circulation time of GEM to <0.3 hours (Erkan et al., 2012; Meng & Nel, 2018).

The pharmacological reduction of stromal cells is one of the main strategies for overcoming pancreatic tumor which is shown by Abraxane®, albumin-bound paclitaxel approved by the Food and Drug Administration (FDA). Clinical studies have revealed that the combination of this drug with GEM improves the survival rate. The underlying mechanism of Abraxane® for the stromal reduction and reduction of the CDA expression is the reactive oxygen species generation (Lancet et al., 2014).

Particle dynamics play an important role in overcoming the stromal barrier (Figure 2) and transportation of nanoparticles from blood vessels to pancreatic tumor cells as a result. Because of the hydrodynamic pressure gradient, an opening temporarily generates through the walls of the blood vessels of pancreatic tumors. Subsequently, fluid enters the pancreatic tumor interstitial space (termed 'eruptions'). Hence, not only 30 nm diameter but also 70 nm diameter nanocarriers can enter into the interstitial spaces of pancreatic tumors. The nanocarriers with 30 nm diameter can rapidly extravasate into the PDAC tumor microenvironment; however, the extravasation of the nanocarriers with 70 nm diameter can be blocked by an abundant dysplastic stroma which can interfere with the drug delivery and cause chemoresistance in pancreatic cancer (Matsumoto et al., 2016). Therefore, concerning tumors with high content, drug delivery systems penetration into the stroma tissue is necessary to reach the tumor cells environment. As a result, the size of delivery systems plays a significant role in the penetrability of the nanocarriers. Many studies showed that smaller delivery systems are preferred to distribute across extracellular matrix with high content of stroma cells (Perrault,

Walkey, Jennings, Fischer, & Chan, 2009). Furthermore, fast growth of pancreatic tumor cells and further compression of blood and lymphatic vessel leads to an increase in fluid pressure throughout the cancer interstitial region, impeding effective penetration of nanocarriers from intravascular region to the pancreatic tumor cells (Kurtanich et al., 2018). Administrating collagenase and transforming growth factor-β inhibitor can respectively decrease the pericyte coverage of endothelium and fibrosis in the pancreatic cancer milieu in order to increase the diffusion of nanocarriers (Samanta et al., 2019). Stromal targeting therapy is another stromal barrier overcoming strategy. Numerous studies have shown that cyclical iRGD peptides bind to tumor-specific integrins. αvβ3 and αvβ5 integrins are then proteolytically processed to reveal a C-terminal (CendR) motif that binds to NRP-1 which could act to induce the formation of grape-like cytoplasmic vesicles and vacuoles, termed the Vesiculo–Vacuolar organelle (VVO) (Ding et al., 2019; Dvorak et al., 1996; Sugahara et al., 2010). This strategy is believed to mediate the transcytosis of nanoparticle-based delivery vehicles into pancreatic tumor cells (Liu et al., 2017).

Figure 2. Schematic illustration of extracellular barriers in pancreatic cancer siRNA delivery.

It is of note that the previous mentioned size of nanoparticle-based delivery systems must be maintained even in the blood circulation containing various cells and biomacromolecules. Therefore, nanocarriers should be rigorously developed to minimize fast dissociation and unwanted aggregation in the biological environment. In particular, the nanoparticle-based delivery systems with positive charge can electrostatically interact with proteoglycans and proteins with anionic charge in the serum, like heparan sulfate and albumin, leading to their dissociation and/or aggregation (Hui et al., 2019). In an important study, transmission electron microscopy (TEM) has demonstrated that the CALAA-01 in blood (with a zeta-potential of 10–30 mV) can be filtered through GBMs due to the aggregation with a high density of heparan sulfate. Therefore, the structural integrity of several delivery systems will be lost. On the other hand, it has been reported that the excretion of siRNA-conjugated cationic polysaccharide nanocarriers is considerably lower than siRNA without delivery systems. Although the initial size of the delivery systems (220-230 nm) was bigger than the GBMs pore size, some siRNAs were excreted through the urine. This suggests that siRNA payloads were slowly disengaged from the nanoparticles. Furthermore, it seems that GBM slightly enhances disassembly of the nanocarriers through IV route (Naeye et al., 2013; Zuckerman, Choi, Han, & Davis, 2012). It is noteworthy that large nanocarriers, <300 nm, can be captured and phagocytosed by Kupffer cells and removed from body (Gustafson, Holt-Casper, Grainger, & Ghandehari, 2015). Hence, the opsonization of nanoparticles can make them more accessible to phagocytes. A robust strategy to limit nanoparticle opsonization by serum proteins is surface modification with hydrophilic, non-ionic, and flexible polymer chains such as poly(oxazoline), poly(N-(2-hydroxypropyl) methacrylamide) (PHPMA), poly(Nvinyl pyrrolidone) (PVP), and polyethylene glycol (PEG), which all improve colloidal stability of nanoparticle-based delivery systems and hinder the non-specific interactions with serum protein (Adler & Leong, 2010; J. Hu et al., 2018). Among these polymers, PEG, an injectable biocompatible, hydrophilic, and biologically-inert material, is the most typically utilized polymer for nanoparticle modification and it is approved by FDA in United States for numerous applications (Adiseshaiah, Crist, Hook, & McNeil, 2016).

Intracellular barriers

The cellular uptake is the first intracellular barrier that the delivery systems encounter (Figure 3). Nanoparticle-based delivery systems should interact with components of the outer surface of cells for internalization into them. To date, five recognized mechanisms have been proposed for the uptake of nanoparticles based on the proteins involved in the endocytosis process; micropinocytosis, phagocytosis, caveolin-mediated, clathrin-mediated, and clathrin/caveolin-independent endocytosis (Sahay, Alakhova, & Kabanov, 2010; Q. Sun et al., 2019). The central parameters determining the endocytic pathway are morphology, surface chemistry, and size. These factors can affect both cellular uptake efficiency and the endocytic route (J. Zhao & Stenzel, 2018). To improve the EPR effect, 30-100 nm-sized multimolecular nanoparticle-based delivery systems have been typically designed, including polymeric micelles and lipid nanoparticles (LNPs). Moreover,

size of rigid nanoparticles affects their endocytosis; 20-50 nm-sized bare gold nanoparticles have the highest cellular uptake in pancreatic cancer cells (H. Gao, Shi, & Freund, 2005). Surface chemistries of nanoparticle-based delivery systems are much more important for their endocytosis, in comparison with shape and size. Cationic nanoparticle-based drug delivery systems have a high affinity for anionic proteoglycans presented on the membrane of various cells, leading to more effective adsorptive endocytosis than anionic and neutral nanoparticle-based drug delivery systems. It is noteworthy that heparan sulfate proteoglycans, consisting of transmembrane proteins known as syndecans, are regarded as the main binding sites for positively charged nanoparticle-based drug delivery systems (Zhi et al., 2018). But the excess positive charge can lead to some serious problems such as rapid opsonization and clearance, toxicity, and increased immunological reactions. To reduce aggregation of particles and consequently overcome the aforementioned problems, PEGylation of nanoparticle-based drug delivery systems is a common approach (Suk, Xu, Kim, Hanes, & Ensign, 2016). Nevertheless, PEGylated delivery systems still have some limitations, including immunogenicity and low cellular uptake and endosomal escape (Lai & Wong, 2018).

To address these limitations, a vast amount of research devoted to the design of nanocarriers with cell recognition moieties (ligand-mediated targeting strategies) to improve their cellular uptake through receptor-mediated endocytosis. On the other hand, these nanoparticles can be designed to lose their PEG-shell at cell surfaces or in the acidic endosomes of cells to increase their release into the cytoplasm (Öztürk-Atar, Eroğlu, & Çalış, 2018).

Entrapment of nanoparticle-based siRNA delivery systems into the vesicles, including early endosome (pH 6.5), late endosome (pH 6.0), and lysosome (pH 4.5–5.0), often leads to their breakdown due to their susceptibility to acidic environment and the involved enzymes (S. A. Smith, Selby, Johnston, & Such, 2019). Hence, the design of delivery systems should facilitate its entry from vesicles into the cytoplasm (Figure 3). Numerous works have established that polycations comprising amines with low pKa and their polyion complexes (PICs) with siRNAs can induce endosomal escape via two mechanisms. First, some of these low pKa amines have a role in electrostatic interaction with anionic siRNAs whereas the protonation of unbound amine groups can take place in acidic pH of the endosomal compartments (B. S. Kim et al., 2019). Through this process, influx of protons along with chloride ions is induced. As a result, the osmotic pressure increases in endo/lysosomal vesicles inducing the endosomal disruption defined as the "proton sponge effect". Another proposed mode of action is that these highly charged polycations can directly destabilize endosomal membrane (Koide et al., 2019). As formerly mentioned, polycations can interact with the negatively charged cytomembrane and disrupt membrane integrity. Polycations, which bear low pKa amines can considerably increase their cation density via amine protonation in endo/lysosomal vesicles and thus destabilize the membrane of endo/lysosome.

Figure 3. Schematic illustration of intracellular barriers in pancreatic cancer siRNA delivery.

Nanoparticles-based delivery systems for pancreatic cancer siRNA therapy

Lipid-based delivery systems

Liposomal nanoparticles have been traditionally the most typically used nanoparticle-based vehicles for siRNA delivery to pancreatic tumor tissues. Despite great transfection efficiency and capacity to form complexes with negatively charged siRNA, cationic lipids/liposomes are extremely toxic due to the induction of reactive oxygen species (ROS) and high intracellular calcium levels (Zahednezhad, Saadat, Valizadeh, Zakeri-Milani, & Baradaran, 2019). Cationic lipids also have a role in complement system activation and thus lead to their rapid removal by reticuloendothelial system (e.g. macrophages). It was also shown that cationic lipids also have a high toxicity to macrophages and other cells of immune system. On the other

hand, anionic liposomes are rapidly cleared from the blood (Y. Zhu et al., 2019). As a result, precise selection of lipids and formulation strategies may help decrease the potential toxicities.

Arsenic trioxide (ATO) is one of the most effective compounds in the treatment of cancer but one of the bottlenecks for its clinical use is its toxicity to normal tissues (Aslan, Shahbazi, Ulubayram, & Ozpolat, 2018). To solve this problem Zeng *et al.* (Zeng et al., 2014) designed a platform of poly (ethylene glycol)-block-poly (DL-lactide) (PEG-PDLLA) to deliver arsenic. Moreover, a novel polymer structure, Poly (ethylene glycol)-block-poly (L-lysine) (PEG-PLL), was introduced to enhance antitumor activity both *in vitro* and *in vivo,*by means of the coadministration of mutant K-ras siRNA (PEG-PLL/siK-ras). This coadministration with PEG-PLL/siK-ras seems to reduce cell growth, migration, and invasion as well as colony formation. Also, there was a dramatic increase in the percentage of PANC-1 cells in the G0/G1 phase compared with S phase. In addition, the synergistic effect of siK-ras and arsenic could downregulate Bcl-2 and K-ras protein expression and reduced tumor growth with higher cytotoxicity.

Survivin is one of the overexpressed proteins in human pancreatic cancer that is related to more extensive metastases, chemo/radio-resistance, and shorter median survival. Although usage of paclitaxel can expand the tumor interstitial space by induction of apoptosis, it can significantly increase the survivin protein level in residual tumors. The cotreatment of this drug and gene therapy was proven to be effective (Satoh et al., 2001). Wang and colleagues (J. Wang et al., 2015) examined this treatment using the paclitaxel and pegylated cationic (PCat)-siRNA lipoplexes (PCat-siSurvivin) in human pancreatic Hs766T xenograft tumor. Their combinational application could significantly improve the efficacy of tumor regression and delayed tumor regrowth.

Regarding the importance of CUX1 transcription factor in the regulation of cell proliferation and differentiation and its possible roles in tumorigenesis and tumor progression, a study by Ripka and colleagues (Ripka et al., 2010) showed that polyethyleneimine (PEI)-complexed siRNA against CUX1 (siCUX1) can increase apoptosis induction in PANC1 and ImimPC1 pancreatic cancer cell lines; via GEM and 5-FU and TRAIL pathway. Furthermore, following intratumoral injection of PEI-siCUX1, tumor growth in murine xenograft models was inhibited.

Induction of apoptosis is another way of eliminating pancreatic cancer cells. miR-34a is one of the most effective options in this regard (Kurtanich et al., 2019). Hu *et al.* (Q. L. Hu et al., 2013) transferred a new form of this miRNA to different tumor cell lines and mouse models through β-cyclodextrin-polyethyleneimine (β-PEI-CD [PC]) vector. Subsequently, cell population in the G0/G1 phase enhanced in comparison to G2/M and S phases which indicated the inhibition of cell growth and tumor size reduction.

Ubiquitin ligase ITCH plays a significant role in the proteasome-dependent degradation of p73, an important protein in cell cycle arrest and apoptosis pathways (Rossi et al., 2005). Fuente and colleagues (de la Fuente et al., 2015) used poly (propylenimine) dendrimers (DAB-Am16/shSCR) to deliver ITCH targeting short hairpin RNA (shRNA) into MIA PaCa-2 and PANC-1, HPAC and BxPC3 pancreatic cells in addition to MIA PaCa-2 xenograft animal models of BALB/c mice and Sprague-Dawley rats. Results indicated a decrease in the mRNA level of target genes. IV administration of this compound with GEM could reduce tumor volume without much change in body weight.

miR-150 is one of the tumor suppressor miRNAs which can be used as a therapeutic goal but some of its physiochemical features, such as anionic charge, hydrophilicity, sensitivity to nuclease degradation, and inefficient uptake, restrict its use (Kurtanich et al., 2019). Application of Poly (lactic-*co* -glycolic acid)(PLGA) and PEI to deliver miR-150 in human pancreatic cancer cells (Colo-357 and HPAF) are based on measurements of its target genes. This miR-150 delivery showed decreased expression of MUC4 and MUC13 glycoproteins which play roles in pancreatic cancer progression (Kurtanich et al., 2019). Since p53, HER2 and pAKTSer473 expression is affected by MUC13, miR-145 can be considered as one of the treatment options through suppression of MUC13 (Khan et al., 2014). Magnetic nanoparticles formulation (MNPF) for the delivery of miR-145(miR-145-MNPF) showed suppression of MUC13, pAKTSer473, and HER2 expression and returned function of p53 in HPAF-II and AsPC-1 human pancreatic cancer cell lines (Setua et

al., 2017).

Recently, a novel class of nanocarriers termed "lipid–polymer hybrid nanoparticles (LPNs)" has been designed to overcome the challenges of both polymer- and liposome-based delivery systems. The LPNs consists of three main components: (1) a cationic hydrophobic polymeric core in which poorly water-soluble drugs and siRNAs can be encapsulated; (2) a hydrophilic polymeric shell with antibiofouling properties to improve LPNs stability and prolong*in vivo* systemic circulation time; (3) an inner lipid monolayer at the interface of the core and the shell, which play significant role in improving biocompatibility and drug retention of the polymeric core (L. Zhang et al., 2008). There is a large extent of hypoxia in pancreatic cancer tumors. Hypoxia-inducible factor (HIF) transcription factor is one of the crucial factors in the survival of cancer cells in hypoxic conditions. HIF-1α is involved in the regulation of a wide range of genes related to cancer angiogenesis, invasion, and proliferation. Overexpression of this factor can increase the likelihood of resistance to the first-line chemotherapeutic drug such as GEM. Zhao *et al.*(X. Zhao et al., 2015) used LENPs made from cationic e-polylysine co-polymer (ENPs) and EGylated lipid bilayer to deliver HIF-1α siRNA and GEM into PANC-1 human PDAC cells and female BALB/c nude mice model bearing subcutaneous human PANC-1 xenografts. Cytotoxicity and antitumor effects of GEM were significantly increased in the simultaneous application of Gem and HIF-1α siRNA. LENPs showed higher blood stability than ENPs nanoparticles. Moreover, stability of drug in copolymer core, high cellular uptake and cytoplasmic release, significant antitumor effects of the Gem and si-HIF-1α, and immune response reduction suggest using LENP-GEM-siHIF-1α in clinical research.

Polymer-based delivery systems

To date, polymeric carriers have attracted major attention in siRNA delivery system because of their high biocompatibility, low cytotoxicity and their versatility for different modifications to accept desirable properties (S. Aghamiri et al., 2019).

PLGA, which is an FDA-approved biodegradable polyester, has been used to encapsulate siRNA for many years. PLGA poloxamer nanoparticles can be applied to increase loading and transfection efficiency. Pan *et al* . (Pan et al., 2015) demonstrated that targeting hypoxia-inducible factor 2α (HIF-2α), known as endothelial PAS domain protein-1(EPAS1), by siRNA encapsulated in PLGA poloxamer led to better intracellular uptake and reduction of cell viability in pancreatic tumor cells *in vivo* . EPAS1 was indicated to be upregulated in about 67 percent of pancreatic cancer patients (J. Yang et al., 2016). In another study, Guopei and his colleagues (Luo et al., 2009) used siRNA sequence of methyl-CpG binding domain protein 1(MBD1) incorporated into PLGA poloxamer carrier. MBD1 is a transcriptional regulator, which is overexpressed in pancreatic cancer cell lines. They showed that PLGA poloxamer as a non-viral gene vector for MBD1 siRNA can be effectively transfected into BxPC-3 human pancreatic tumor cells and inhibit cell growth and induce apoptosis (Fujita et al., 2003). As an enhancing modification in this common siRNA delivery method, the surface of PLGA is covered by cationic PEI to improve weak electrostatic interaction between PLGA and siRNA.

A number of studies have used Cationic poly (lactic acid) (CPLA), a non-toxic biodegradable polymer incorporated with siRNA to induce the silencing of mutant *K-ras* gene in pancreatic cancer models*in vivo* . Guimiao *et al* . (Lin et al., 2013) designed a new type of carrier with transforming the linear CPLAs into CLPA nanocapsules. These nanocapsules are covalently cross-linked and display several advantages over linear CLPAs such as stronger and steadier scaffold structure. Also, the large surface can be more beneficial for siRNA condensation. Negative charges of siRNA results in its attachment to the surface of CLPA nanocapsules through electrostatic interactions. This novel polymer formulation of CPLA for siRNA delivery was able to transfect over 90% of PANC-1 cells and knockdown *K-ras* gene by almost 50% in PDAC models *in vivo* .

Since 2011, there has been a rapid rise in the use of Star polymers as delivery agents for various therapeutic

purposes (Duong et al., 2014). They can be cost-beneficially produced in large amounts and be modified easily to increase their stability and targets particular cell types. Teo *et al* . (Teo et al., 2016) delivered βIII-tubulin siRNA into mice with orthotopic pancreatic MiaPaCa-2 by poly[oligo(ethylene glycol) methyl ether methacrylate] (POEGMA) via intratumoral administration to inhibit pancreatic tumor growth. The βIII-tubulin siRNA/ POEGMA complexes rendered high transfection efficiency in pancreatic cancer cells *in vitro* and *in vivo* . They also demonstrated that in order to increase the ability of gene silencing and overcome the problem of serum opsonization, cationic nanoparticles PEGylation along with POEGMA (poly [oligo (ethylene glycol) methyl ether methacrylate]) can be used to cover the positive charge.

The use of Poly Glutamic Acid-Based nanocarriers is a potentially promising approach to overcome the existing limitations with the use of RNAi such as low cellular uptake, degradation in the peripheral blood by RNases, clearance by the kidneys and immunogenicity (Huang et al., 2012). Although small RNAs are believed to stimulate cytokine secretion, this type of amphiphilic polyglutamate amine (APA) delivery system does not induce the secretion of TNF-α and IL-6. These nanocarriers show minimal systemic side effects such as accumulation of small amounts in the spleen and low level of immunotoxicity. These nanocarriers are produced by attaching alkylamine and ethylenediamine parts to the carboxylic groups (Han et al., 2018). c-MYC proto-oncogene encodes a transcription factor, which plays a significant role in the regulation of cell proliferation, growth, and apoptosis. MYC oncogene can be targeted by both miR-34a and Polo-like kinases 1 (PLK1). To inhibit this oncogene, a biocompatible APA polymeric nanocarrier has been designed to transfer microRNA-mimic (to enhance miR-34a) and PLK1 siRNA into PDAC-bearing mice through intravenous (IV) route. Results were promising if high miR-34a and low PLK1 expression levels were present; hence, to increase the therapeutic response and survival, restoration of miR-34a together with down-regulation of PLK1 should occur (Gibori et al., 2018).

These studies rationalize the need for continuous development of polymeric based siRNA carriers to pancreatic tumors as well as other solid tumors

Rigid nanoparticles

Rigid nanoparticles, also called inorganic nanoparticles, are more efficient siRNA carriers compared to the soft nanoparticles due to the faster cellular uptake, easier surface modifications, higher stability, higher surface area to volume ratios, and flexible size (Paris, Baeza, & Vallet-Regí, 2019). Here, we summarize a variety of siRNA delivering rigid nanoparticles for biomedical applications in pancreatic cancer.

Gold nanoparticles

Gold nanoparticles (GNPs) can be used not only as a delivery vehicle but also as a therapeutic agent (Hashemi Goradel et al., 2018; Negahdari, Darvishi, & Saeedi, 2019). In several studies, GNPs have been used as effective siRNA carriers, as they encompass various suitable characteristics including surface plasmon resonance property, easily controllable size and shape, and easy modification with other molecules (Y. Gao, Liu, & Li, 2011). GNPs exhibit effective gene knockdown with no detectable toxicity and off-target effects. Recently Gold Nanoclusters (GNCs) with fluorescent tags have gained more attention because of their unique properties. In addition to their ultra-small size, GNCs have fluorescence emission from near-infrared to the visible region (Zheng, Lai, Liu, Jiang, & Wang, 2017).

Perineural invasion is one of the most important pathologic characteristics of pancreatic cancer (Bapat, Hostetter, Von Hoff, & Han, 2011). NGFs (nerve growth factors) are crucial regulators of the tumor-induced neurogenesis and their overexpression has been associated with pancreatic cancer (Z. Zhu et al., 2002). Recently Lei *et al* . (Lei et al., 2017) aimed to develop GNCs to deliver NGF siRNA (GNC–siRNA) in pancreatic cancer. The results showed high silencing of NGF gene and suppression of tumor progression

in three pancreatic tumor models. The GNC–siRNA conjugate showed high stability of siRNA in serum, improved siRNA circulation lifetime in blood, increased cellular uptake, and no apparent toxicity.

Yin *et al* . (Yin et al., 2015) used layered structure of gold nanorods (AuNR) with anionic PSS polymer coating to gravitate doxorubicin and on the other hand, cationic PAH polymer was used to take up K-ras siRNA (AuNRs/DOX/K-ras). This compound inhibits tumor growth for at least 25 days through the controlled release of doxorubicin and K-ras siRNA in cancer cells and subsequent laser light exposure.

Iron oxide nanoparticle

Superparamagnetic iron oxide nanoparticles (SPIOs) have been employed as drug carriers and contrast agents for MRI due to their biocompatibility and intrinsic magnetic properties. SPIOs typically consist of an iron oxide core and a hydrophilic shell. An important obstacle that limits the use of SPIOs is their inability to reach sufficient concentration at the tumor site (Dulińska-Litewka et al., 2019). Mahajan *et al* . (Mahajan et al., 2016) used SPIOs coupled with siRNA to target PLK1, an important oncogene in pancreatic cancer. SPIOs were coated with dextran to be detectable by MRI and were further conjugated with streptavidin (StAv-SPIONs). Later, they were also coupled by myristoylated polyarginine peptides to enhance intracellular delivery alongside endosomal escape and a non-immunogenic tumor-selective peptide (EPPT1) to specifically target the tumor antigen. The results revealed that these siPLK1-StAv-SPIOs can efficiently silence PLK1 expression and stop tumor progression in both *in vitro* and *in vivo* models. The delivery of siPLK1-StAv-SPIOs to the tumor site was increased, which was qualified by MRI. siPLK1-StAv-SPIOs showed lower cell toxicity compared with StAv-SPIOS without an active bioligand.

Magnetic nanoparticles can be used to convert nanoparticles into theragnostic multifunctional delivery systems in order to achieve effective real-time imaging of pancreatic cancer tissue with high-resolution.

Calcium phosphate nanoparticles

Calcium phosphate (CaP) has been utilized as an efficient gene vector for nearly 40 years and siRNA carriers for recent years. Due to their homology to bone and teeth, CaPs have non-toxic nature and exhibit biocompatibility, biodegradability, and lack of immune stimulation. However, one of their major limitations is the untunable growth of the CaP crystal reducing the transfection efficiency (Habraken, Habibovic, Epple, & Bohner, 2016). To address this problem, Pittella *et al* . (Pittella et al., 2011) prepared a hybrid nanoparticle system to deliver siRNA to pancreatic cancer cells (PANC-1). This hybrid system is made of charge conversional polymer (CCP) as siRNA vehicle, and CaP as the block copolymer of PEG. In this system, CaP constitutes a stable core for siRNA and PEG–CCP encapsulation. The non-toxic synthesized PEG–CCP exerts strong membrane destabilization and rapid escape of siRNA. The incorporation of PEG-block siRNA into CaP leads to a size-controllable hybrid nanoparticle and facilitates the cellular uptake. Encapsulated siRNA (CaP-siRNA with PEG-block polymer nanocarrier) showed high knockdown of VEGF in PANC-1 cells compared with naked siRNA.

Silica nanoparticles

Mesoporous silica nanoparticles (MSNP) with high surface area and channels with porous have gained increasing attention in the past decades. Besides their well-developed surface chemistry, silica materials have several advantages including safety, biodegradability, and biocompatibility which make them suitable for efficient drug delivery platforms and siRNA carriers (Y. Wang et al., 2015). Xia*et al* . (Xia et al., 2009) showed that PEI-coated MSNPs exhibited high cellular uptake (up to 70%) and high binding efficiency to DNA and siRNA. They also reported that the cytotoxicity and delivery efficiency can be modified by

adjusting PEI chain length. They found that MSNPs which are coated with PEI polymers of low molecular weight enhance the delivery of paclitaxel (an anticancer drug) to pancreatic cancer cells (PANC-1).

Graphene oxide nanomaterials

Graphene oxide (GO), a common derivative of graphene, is an exciting nanomaterial that possesses several physical properties, which make it suitable for biomedical applications. GO is water-soluble providing large platform for convenient functionalization, and it exhibits fluorescent emission in near-infrared (NIR) to the visible spectrum (Maiti, Tong, Mou, & Yang, 2019). Yin *et al* . (Yin et al., 2017) proposed a multi-functionalized monolayer system by folic acid (FA) and Poly-allylamine hydrochloride (PAH) conjugation onto GO nanosheets. This allows for dual gene silencing in pancreatic cancer cells (MIA PaCa-2). It was ascertained that *HDAC1* and *K-ras* , two genes essential for cell growth, were silenced simultaneously and the transfection efficiency was over 90%. Furthermore, as a result of the synergic combination of gene silencing and photothermal activity of GO under NIR light, *in vivo* cell proliferation was inhibited by >80%. The system exhibited low toxicity and may serve as a highly biocompatible nanocarrier for biomedical applications.

Specific ligand delivery

Some receptors have a specific or excessive expression on cancer cells. Binding molecules to nanoparticles that recognize cancerous cells via these receptors can improve the effectiveness of treatment. In addition, active targeting of delivery systems can be enhanced by tumor receptor-mediated targeting. Specific ligands for siRNA delivery to the different types of cancer cells include hyaluronic acid (HA), transferrin, antibody, single-chain variable fragment (scFv), EphrinA I, anisamide (AN), follicle-stimulating hormone (FSH), folic acid (FA), Epidermal growth factor receptor (EGFR), gastrin, octaarginine, and arginyl-glycyl-aspartic acid (RGD) (Aghamiri, Jafarpour, Malekshahi, Mahmoudi Gomari, & Negahdari, 2019). Of these, only antibody, gastrin, and RGD have been considered for siRNA delivery in pancreatic cancer among them, which have been completely review in this part.

RGD

RGD peptides are common ligands used for the delivery of siRNA into cervical cancer cells due to the fact that they are directly related to cancer angiogenesis and metabolism of quick-proliferating pancreatic tumor cells. The RGD peptide can robustly and selectively interact with overexpressed receptors of αvβ5 and αvβ3 integrins on pancreatic cancer cells (Y. Sun et al., 2017). Kim *et al* . (H. A. Kim, Nam, & Kim, 2014) designed PA-PEG1k-RGD to deliver VEGF siRNA in PANC-1 cells. The PA-PEG1k-RGD/VEGF siRNA complexes showed higher cellular uptake and gene silencing than non-targeted ones.

Antibodies

Monoclonal antibodies and their fragments have been widely used for treatment and diagnosis of various diseases (Suurs, Lub-de Hooge, de Vries, & de Groot, 2019). Antibody-based deliveries offer a robust strategy to develop novel specific ligand-coupled nanoparticle systems for siRNA delivery (Kulhari, Jangid, & Adams, 2019). The antibody structure can be categorized into two distinct biofunctional parts. The crystallizable fragment (Fc) binds to Fc receptor on the membrane of the immune cell or other antibodies and the antigen-binding fragment (Fab) mediates antigen recognition via complementarity-determining regions.

Fab fragments are useful components for decreasing the size of antibodies (around 15 nm), and thus reducing immunogenicity and increasing the pharmacokinetic profile of nanoparticle-based drug delivery systems (Warram et al., 2014).

Human tissue factors (TFs) are overexpressed in pancreatic cancer cells. Thus, anti-human TF can be used to increase the efficacy of delivery (Chen et al., 2014). Min *et al* . (Min et al., 2018) developed polyion complex (PIC) micelles which were composed of a copolymer of azide-functionalized polyethylene glycol and poly-L-Lysine and further modified with anti-human tissue factor (TF) in order to deliver PLK1 siRNA into the pancreatic cancer BxPC3 cells. Gene silencing activity against PLK1 mRNA in cancer cells was the most significant in the 3(Fab')-micelle. Moreover, a higher penetrability and the cellular internalization amount in BxPC3 spheroid was observed with the 3(Fab')-micelle compared with one or two molecule(s) of Fab'-conjugated PIC micelles.

Li *et al.* (Li et al., 2016) designed Iron oxide nanoparticles (IONPs)-PEI delivery system modified with anti-human CD44v6 single-chain variable fragment ($scFv_{CD44v6}$) to transfer GEM and siRNA targeting Bmi-1 (B-cell-specific Moloney murine leukemia virus insertion site 1) which play important roles in proliferation, survival, and invasion of pancreatic tumor cells. The GEM-scFv-IONP-PEI/siBmi-1 structure shows a synergistic anti-pancreatic tumor effect not only*in vitro* but also *in vivo* .

Gastrin

Gastrin tends to interact with the overexpressed cholecystokinin-B (CCK-B) receptor with a high affinity in various types of pancreatic cancer cells (Banerjee & Saluja, 2018). Therefore, to target gastrin mRNA in pancreatic cancer cells, some researchers use gastrin-mediated delivery. In one of these studies, gastrin-polyethylene glycol-poly(L-lysine) (Ga-[PEG]5k-PLL27) has been designed by Burks and his co-workers (Burks et al., 2018) to deliver gastrin siRNA into the pancreatic tumor cells. The Ga-conjugated carrier revealed greater gene silencing of gastrin in PANC-1 cells than unmodified ones.

Modes of actions of MDR

Pump resistance

One of the main mechanisms of MDR is pump resistance which is associated with membrane efflux pumps and reduced anticancer drug accumulation in cells (Nikolaou, Pavlopoulou, Georgakilas, & Kyrodimos, 2018). Multidrug efflux pumps are categorized into MDR associated-protein-1 (MRP1) and P-glycoprotein. P-glycoprotein is a member of superfamily ATP-dependent transporter that acts as an detoxifying agent and ejects toxin and xenobiotic by pumping positively or neutral charged chemotherapy medications out of the cells (e.g. vincristine, etoposide, doxorubicin (DOX) and actinomycin D) (Robey et al., 2018). MRP1 belongs to the ATP-binding cassette (ABC) transporter superfamily that can pump out various compounds instantiating anionic, hydrophobic, glutathione-conjugated (GSH) anticancer drugs and fluorescent dye (e.g. sulfate conjugates, folates, glucuronide conjugates, heavy metal anions and toxicants and etc.) (Nanayakkara et al., 2018). Various factors contribute to the occurrence of MDR in pancreatic cancer like blocking of apoptotic pathways, accelerated drug metabolism and DNA repair, reduced drug uptake, metabolic changes, and the presence of chemotherapy-resistant cancer stem cells. ABC transporters play a central role in the reduction of intracellular accumulation of drugs and resulted pancreatic cancer MDR (Figure 4).

Figure 4. Schematic illustration of pump resistance and PLK1-mediated nom-pump resistance mechanism.

Non-pump resistance

Non-pump resistance is not dependent on the drug efflux pump, and it is based on non-pump proteins such as superfamily Bcl-2, VEGF, and PLK1 which contributes to escape of cancer cells from apoptosis (W. Zhu, Shan, Wang, Shu, & Liu, 2010) (Figure 4). Non-pump proteins provide resistance of cancer cells through modifying the target cell of the drug, activating the DNA repair system, detoxifying system (the cytochrome p450 mixed-function oxidase), activating antioxidant pathways such as reactive oxygen species (ROS), changing in cell-cycle checkpoints signals, prohibiting the onset of apoptosis and blocking it. Bcl-2, as the first anti-cell death protein, contains anti-apoptotic (Bcl-XL, MCL-1, Bcl-w, Bcl-2, etc.) and pro-apoptotic (Bak, Bid, Bax, Bad, etc.) members. PLK1 has been investigated as a pancreatic cancer drug target, which caused by *K-ras* mutations (S. Kumar & Kim, 2015). Many studies established that silencing PLK1 gene expression can lead to *K-ras* mutated pancreatic cancer cell death (S. Kumar, Sharma, Sharma, Chakraborty, & Kim, 2016). As a consequence, co-delivery of PLK1 siRNA and chemotherapeutic agents has been extensively studied as a novel promising approach for the treatment of various types of cancer (Yu et al., 2019) (Figure 5).

In recent years, the development of innovative multifunctional delivery systems offers a new potential therapeutic avenue for pancreatic cancer including both pump and non-pump resistance genes targeting, through co-delivery of siRNAs and chemotherapeutic agents together with imaging (Figure 5).

Figure 5. A new potential therapeutic avenue for pancreatic cancer including both pump and non-pump resistance genes targeting, via co-delivery of siRNAs and chemotherapy agents, and imaging.

Co-delivery of chemotherapy agents and siRNA for anti-MDR pancreatic cancer therapy

Despite increasing understanding of the mechanisms underlying chemoresistance in pancreatic cancer, the therapeutic potential of their pharmacological inhibition has not been successfully exploited yet.

Recently, the combination of nanoparticle-based delivery systems, siRNAs, and chemotherapy drugs has emerged as a robust strategy for cancer therapy (Godsey, Suryaprakash, & Leong, 2013) (Figure 5). Examples of co-delivery of chemotherapeutic agents and siRNAs for the pancreatic cancer therapy are listed in Table 3.

To deliver anti-HER-2 siRNA, Pirollo *et al* . (Pirollo et al., 2007) designed an anti-transferrin receptor (TfR) single-chain antibody fragment-directed nanoimmunoliposome (scL) and then intravenously injected in the mouse model bearing subcutaneous human PANC-1 xenografts. Smaller tumor size was reported in mice treated with scL-HoKC/HER-2 siRNA plus GEM than mice treated with GEM alone.

Fuente *et al* . (de la Fuente et al., 2015) used third-generation poly(propylenimine) dendrimers (DAB-Am16) to transfer anti-ITCH siRNA and shRNA in MIA PaCa-2 and PANC-1 cells and mice bearing MIA PaCa-2 xenografts. The dendriplexes/anti-ITCH siRNA and shRNA complexes showed high cellular uptake and gene silencing *in vitro* ; and when co-delivered with GEM demonstrated great efficiency in gene knockdown against mice bearing PaCa-2 xenografts via i.v. administration. This co-delivery strategy also increases the chemosensitivity of pancreatic cancer cells.

Notch1 has a central role in the regulation of cell differentiation, proliferation, survival, and maintenance of various types of cancer cells (Paryan et al., 2016; Takebe, Harris, Warren, & Ivy, 2011). Yang*et al* . (C. Yang et al., 2017) co-delivered K-ras and Notch1 siRNA and GEM into MiaPaCa-2 cells using biodegradable charged polyester-based vectors (BCPVs). Cotreatment of BCPV-siRNAK-ras-siRNANotch1 nanocomplexes and GEM significantly reinforced antitumor efficacy, apoptosis, and also reversed the epithelia-mesenchymal transition (EMT) with high efficacy. Therefore, the combination of siRNA therapy and chemotherapy enhances cellular apoptosis and chemosensitivity.

Conclusion and future perspectives

Nano-siRNA drugs have recently been developed as a promising new class of therapeutic agents to treat different types of cancer including pancreatic cancer. RNAi molecules like siRNA are highly effective therapies for cancer depending on their ability to specifically silence cancer-related genes expression or to selectively regulate the pathways involved in the development and progression of malignancy. The development of MDR is the central problem in the pancreatic cancer therapy approaches. As a potential new strategy against pancreatic cancer, the combination of chemotherapy with nano-siRNA drugs represents a revolutionary solution to overcome MDR. Despite these encouraging advances, the toxicity and immune system stimulation, poor knowledge of nano-bio interactions, and limitations considering designing, manufacturing, clinical translation, and commercialization still remained to be addressed. Moreover, to achieve the best synergistic effect of the combination strategy, the encapsulated siRNA and chemotherapy drugs require to be unloaded in cancer cells at two distinct controlled timings. The drug release profiles for the siRNA and anticancer drug will require to be programmed into the chemistry of the delivery system material in such a way that the siRNA molecules will first be released instantly upon internalization into a cell, and after cancer cell sensitization, the anticancer drug will require to be unloaded from the delivery system. Further innovative ideas will require to be designed and optimized to synthesize an innovative *in vivo* -stable, bio-safe, multi-drug delivery system to overcome the limitations. In recent years, the advent of black phosphorus has proved to be a promising approach for the delivery of gene therapeutics. Hopefully, further studies will be performed to complement the results of this revolutionary research in the near future (Tao et al., 2017).

In summary, nevertheless, the field of nano-siRNA drugs for pancreatic cancer therapy has already come a long way. We believe that nano-siRNA drugs will shift the paradigm of pancreatic cancer therapy and become a reality in the near future.

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Figure 1. Instances of nanocarriers for pancreatic cancer therapy.

Figure 2. Schematic illustration of extracellular barriers in pancreatic cancer siRNA delivery.

Figure 3. Schematic illustration of intracellular barriers in pancreatic cancer siRNA delivery.

Figure 4. Schematic illustration of pump resistance and PLK1-mediated nom-pump resistance mechanism.

Figure 5. A new potential therapeutic avenue for pancreatic cancer including both pump and non-pump resistance genes targeting, via co-delivery of siRNAs and chemotherapy agents, and imaging.

Table 1. Significant siRNA chemical modifications to address siRNA drawbacks.

siRNA modified moiety
Sugar
Backbone Linkage Modifications
Base Modifications
Terminal Conjugates

Abbreviation: 2'-O-Me, 2'-methoxy group substitution; 2'-F, 2'- fluoro substitution; 2'-O-DNP, 2'-O-dinitrophenyl ethers;

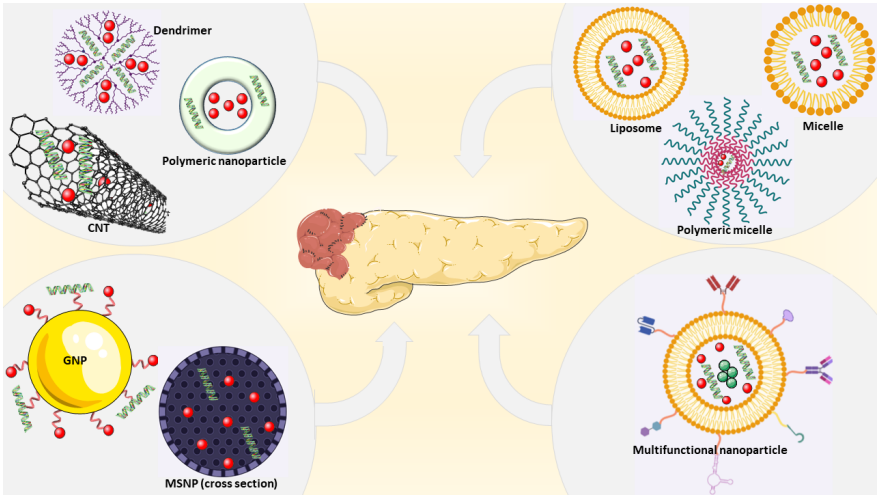
Table 2. nano-siRNA therapeutic agents in clinical trials for the pancreatic cancer therapy.

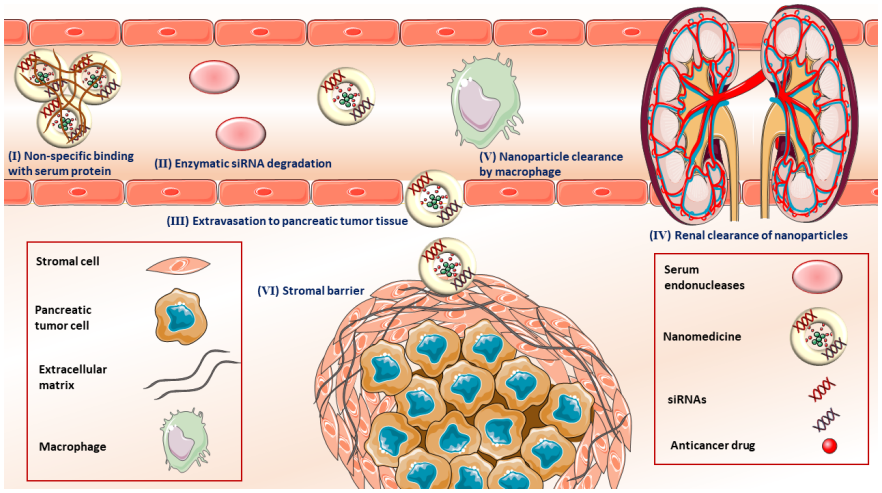
Therapeutic siRNAs/interventions	Targeted gene
siG12D-LODER/ Gemcitabine+nab-Paclitaxel	KRAS-G12D
siG12D LODER	KRAS-G12D
KRAS-G12D siRNA	KRAS-G12D
Atu027 & gemcitabine	PKN3
TKM-080301	PLK1

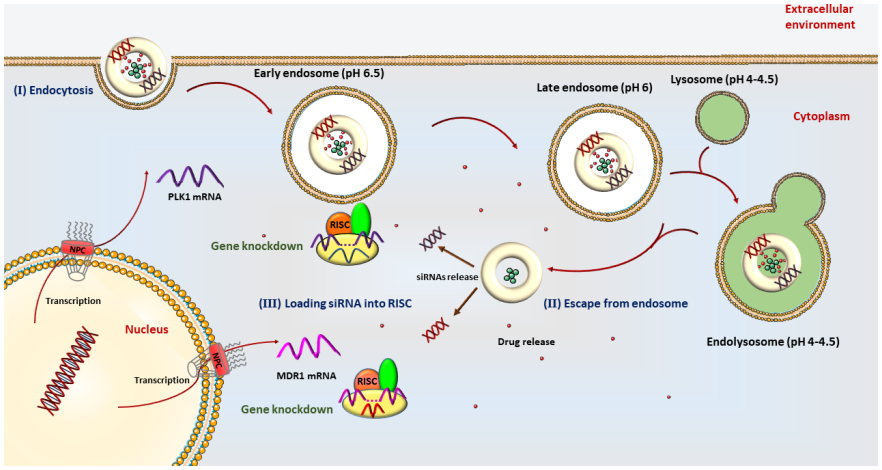
Table 3. Examples of siRNA-based combination therapies for pancreatic cancer.

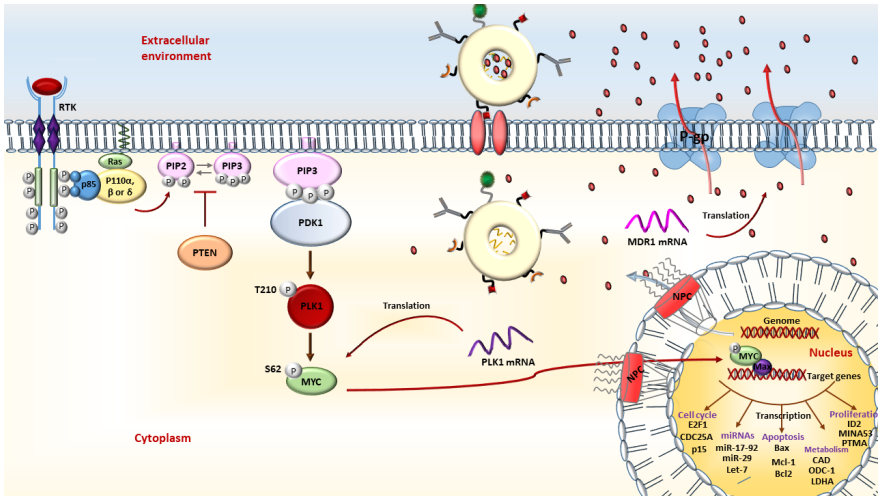
Delivery vehicle
Third generation poly(propylenimine) dendrimers (DAB-Am16)
$scFv_{CD44V6}$- functionalized Iron oxide nanoparticles (NPs)-PEI
polyethyleneimine (PEI)
pegylated cationic liposomes (PCat)
Poly(ethylene glycol)-block-poly(l-lysine) for siRNA and poly(ethylene glycol)-block-poly(dl-lactide) for arsenic
Lipid-polymer hybrid nanoparticles (LENPs)
Polyelectrolyte polymers coated Gold nanorods (AuNRs); therapeutic agents can be released after irradiating with 665 nm l

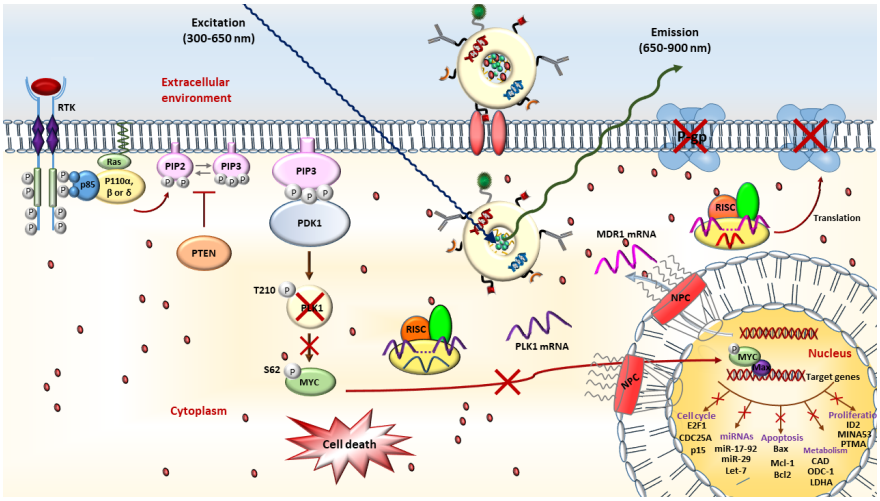
Abbreviation: iv.: Intravenous injection; ip.: Intraperitoneal injection











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